JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

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VOL. 6 1961

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

PRINTED IN THE NETHERLANDS BY
DRUKKERIJ MEIJER N.V., WORMERVEER AND AMSTERDAM

EDITORIAL

When the number of volumes of the *Journal of Chromatography* was changed from one to two per year, it was hoped that the publication time lag would be reduced to the order of a few months. However, the unexpectedly large number of papers received has since increased the waiting time to well over six months. It has therefore been decided to publish three volumes of the *Journal of Chromatography* during 1962; by this means we hope not only to keep the waiting time of manuscripts within reasonable limits, but also to expand the data section for which much material has also begun to accumulate.

Beginning with volume 6 there will be two categories of short communications. Short papers, descriptions of apparatus, etc., will from now on be referred to as "Notes", while only preliminary results of an urgent nature will be called "Short Communications" and these will be published with the minimum delay.

REVUE D'ENSEMBLE

PROGRÈS RÉCENTS DE LA MICROCHROMATOGRAPHIE SUR COUCHES MINCES

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(Reçu le 1 mars 1961)

La présente revue d'ensemble complète et met à jour un premier article consacré au même sujet en 1958[1]. Depuis lors, la microchromatographie sur couches minces a connu un important développement. Rendue plus accessible sur le plan pratique, elle a été adaptée, avec un succès remarquable, à l'analyse des substances hydrosolubles. Son usage se généralise de plus en plus.

I. APPAREILLAGE, TECHNIQUE

(*a*) *Préparation des couches adsorbantes*

Seules les chromatoplaques préparées à l'aide d'égaliseurs produisant des couches actives rigoureusement régulières et d'épaisseur constante assurent une reproductibilité satisfaisante des résultats. L'un de ces appareils, construit par STAHL[2], donne des couches adsorbantes de 0.01 à 0.5 mm d'épaisseur. Il en existe un modèle commercial* produisant des couches de 0.25 mm à partir de mélanges fluides d'adsorbant, de plâtre de Paris et d'eau. Un mode d'emploi détaillé libère l'utilisateur de cet appareil de toute difficulté pratique concernant la préparation proprement dite des chromatoplaques; ce facteur de commodité a largement contribué à la diffusion de la méthode ces deux dernières années.

STAHL[2] a mis au point un autre appareil (non commercialisé), électrique, permettant la préparation automatique de 800 à 1500 "chromatostrips" (2.5 × 20 cm) par heure avec réglage variable de l'épaisseur des couches adsorbantes (de 0 à 1 mm).

Un égaliseur plus simple, construit en plexiglas, a été décrit par BARBIER *et al.*[3] pour la production de couches adsorbantes régulières de 0.3 mm d'épaisseur environ.

MACHATA[4] a construit un instrument tout aussi simple, en acier inoxydable, donnant un film adsorbant de 0.25 mm environ.

MOTTIER[5] a utilisé de son côté un dispositif encore plus simple pour répartir régulièrement de l'alumine pulvérulente sur des plaques de verre. Il s'agit là toutefois d'une technique spéciale conduisant à des couches adsorbantes non adhérentes et non à des chromatoplaques au sens habituel du terme.

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* Fabriqué par C. Desaga GmbH, Hauptstrasse 60, Heidelberg (Allemagne). Un autre égaliseur est construit par Camag S.A., Muttenz (BL/Suisse).

A l'exception de l'appareil réglable de STAHL[2], tous ces égaliseurs donnent des couches d'une épaisseur unique comprise entre 0.2 et 0.3 mm, limites adoptées par la quasi-totalité des utilisateurs de chromatoplaques. On ne doit cependant pas perdre de vue l'intérêt que présente l'emploi des couches spécialement épaisses (1 mm) qui autorisent les séparations semi-préparatives et méritent particulièrement bien le nom de "colonnes ouvertes" conféré aux chromatoplaques en général. Il ne semble par contre guère y avoir d'avantage à utiliser des couches adsorbantes extrêmement minces (<0.2 mm) sur lesquelles la reproductibilité des valeurs de R_F est influencée par les plus petites irrégularités de l'adsorbant[2].

Les chromatoplaques préparées sans l'aide d'égaliseur conduisent à des résultats utilisables si l'on caractérise les substances chromatographiées par leurs valeurs de *R_F relatives*. Celles-ci restent en effet, dans certaines limites, indépendantes des variations d'épaisseur des couches. Citons à ce propos TSCHESCHE *et al.*[6] qui ont utilisé des chromatoplaques préparées simplement à l'aide d'une spatule.

(*b*) *Composition des couches adsorbantes*

L'adsorbant le plus généralement utilisé sur chromatoplaques est l'acide silicique (silicagel). Ses propriétés chromatographiques à la fois souples et sélectives et le fait qu'il peut être adapté aussi bien à la séparation de corps liposolubles (par adsorption) qu'à celle de corps hydrosolubles (par partage) justifient cette préférence. L'oxyde d'aluminium, d'un emploi beaucoup plus restreint, paraît surtout recommandé pour les séparations des substances basiques. MOTTIER[5] a cependant obtenu d'intéressants résultats avec cet adsorbant dans le cas d'acides aminés et de colorants.

Il existe, dans le commerce, des adsorbants préparés tout spécialement pour l'emploi sur chromatoplaques*. Ces produits contiennent une certaine quantité (par exemple 5%) de plâtre comme agent liant.

PRYOR ET BRYANT[7] ont préféré le magnésol (silicate hydraté de magnésium) à l'acide silicique dans le cas de la microchromatographie de certaines huiles essentielles.

Dans le domaine particulier de la chromatographie de partage, TEICHER *et al.*[8] ont mentionné l'usage d'une poudre de cellulose commerciale** sur chromatoplaques. Rappelons que la série "G" des adsorbants de Merck comprend le kieselguhr.

L'emploi d'autres adsorbants s'imposera certainement à l'avenir, particulièrement dans le but de permettre des séparations spéciales (couches minces à base de polyamides, de résines échangeuses d'ions, etc.).

L'agent liant incorporé à l'adsorbant est presque toujours le plâtre, ce qui donne des couches actives exclusivement minérales supportant l'action des réactifs de révélation les plus corrosifs. L'amidon constitue un autre excellent liant, de moins en moins utilisé cependant à cause de sa nature organique. Il conduit pourtant à des couches adsorbantes réellement dures, permettant des inscriptions au crayon, ce qui n'est pas le cas, par exemple, de celles préparées à partir du silicagel "G" (Merck).

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* Silicagel "G", oxyde d'aluminium "G" et kieselguhr "G" fabriqués par E. Merck, Darmstadt (Allemagne); "G" = Gips = plâtre. Un oxyde d'aluminium pour chromatographie sur couches minces est également fabriqué par Fluka S.A., Buchs, St.-Gall. (Suisse).

** Fabriquée par Excorna, Pharm. Präparate o.H.G., Mainz (Allemagne).

(*c*) *Normalisation des couches adsorbantes*

Les films actifs utilisés pour la microchromatographie doivent respecter certains critères que STAHL[2] s'est attaché à bien définir. Cet auteur tient compte de trois propriétés fondamentales des chromatoplaques pour fixer les normes suivantes, auxquelles doivent répondre les couches siliciques de 0.25 mm d'épaisseur activées à 105° durant 30 minutes.

1. *Pouvoir séparateur, degré d'activité.* En développant avec de l'hexane le microchromatogramme d'un mélange de trois colorants (bleu d'indophénol, rouge Soudan G, *p*-diméthylaminoazobenzène; 2 μg de chaque), on doit constater que ceux-ci restent à leur point de départ (situé à 1.5 cm du bord inférieur de la plaque, celle-ci trempant de 0.5 cm dans le solvant). L'essai est terminé lorsque le front du solvant a atteint un niveau situé à 10 cm au-dessus de la ligne de départ.

En répétant le même essai en présence de benzène, on doit observer une migration et une sèparation parfaites des trois colorants. Le degré d'activité exact de la plaque peut être chiffré par les valeurs de R_F obtenues.

2. *Propriétés capillaires.* Le temps mis par le benzène pour couvrir un trajet ascendant de 10 cm sur la couche adsorbante, dans les conditions normales de la chromatographie, ne doit pas excéder 45 minutes (30 minutes habituellement).

3. *Structure physique, résistance mécanique.* L'adsorbant doit posséder un degré granulométrique permettant l'obtention de couches régulières, d'apparence lisse, de 0.25 mm d'épaisseur. Ces couches doivent pouvoir supporter une forte pulvérisation d'acides concentrés suivie d'un chauffage.

Le silicagel "G" (Merck) satisfait à ces exigences. Il est toutefois recommandé de placer un témoin (par exemple le mélange des trois colorants cités plus haut) sur chaque chromatoplaque utilisée. On obtient alors un maximum de précision, les plaques d'activité anormale pouvant être décelées et leurs résultats corrigés. Cette pratique est d'autant plus justifiée que l'obtention d'une reproductibilité parfaite des valeurs de R_F absolues nécessite de grandes précautions expérimentales difficiles à observer pleinement (manipulation des plaques à l'abri total de l'humidité atmosphérique, utilisation de cuves idéalement conditionnées pour le développement, etc.). Dans les conditions les meilleures, les valeurs de R_F sont constantes à $\pm$ 0.05 près.

L'un des principaux avantages de la microchromatographie sur couches minces est de permettre, à partir de très peu de produits, l'étude des conditions les meilleures à adopter (choix du solvant, etc.) pour effectuer des séparations préparatives sur colonnes. Ce passage de l'échelle micro- à l'échelle macrochromatographique ne soulève pas de problème particulier pour un même adsorbant, bien que le pouvoir de définition des colonnes s'avère souvent relativement amoindri par des effets de diffusion.

STAHL[2] a constaté que les couches adsorbantes préparées à partir du silicagel "G" (Merck) possèdent un pouvoir adsorbant "correspondant" à celui de l'oxyde d'aluminium neutre d'activité II/III (Merck) (détermination d'après BROCKMANN ET SCHODDER[9]). Une telle correspondance des deux adsorbants ne peut être valable que dans un nombre limité de cas. STAHL[10] a d'ailleurs constaté que les valeurs de R_F

obtenues sur l'un et l'autre pouvaient différer, particulièrement en présence de mélanges de solvants. Rappelons que l'adsorption sur acide silicique s'effectue principalement par liaisons hydrogène entre les groupes silanols et les groupements polaires des molécules du soluté[11], alors que l'oxyde d'aluminium paraît agir, dans une plus large mesure, par d'autres facteurs[12] (forces de Van der Waals, pouvoir échangeur d'ions, etc.). De cette diversité dans les modes d'adsorption, il ressort que la reproduction à l'échelle préparative des séparations obtenues sur chromatoplaques ne peut parfaitement s'effectuer que sur des colonnes de même adsorbant.

La forme des "chromatostrips" exerce une certaine influence sur les valeurs de R_F, ainsi qu'il résulte d'une étude de FURUKAWA[13]. L'intérêt de ce phénomène, qui ne semble pas avoir d'incidence pratique, paraît toutefois assez secondaire.

(*d*) *Choix du solvant de développement*

La règle, en microchromatographie d'adsorption, est d'accorder la polarité du solvant à celle du mélange à séparer. On s'adressera donc à la série dite "éluotrope" pour effectuer un premier choix. Une détermination plus précise du meilleur solvant peut ensuite être faite par la microtechnique circulaire de STAHL[2]: on dépose, à quelques centimètres d'intervalle sur une couche adsorbante, plusieurs gouttes d'une solution du mélange à chromatographier. Après évaporation du solvant, on dépose au centre de chacune des taches un fin capillaire rempli d'un des solvants à examiner. Chaque tache subit un développement radial très rapide et l'examen de la plus ou moins grande netteté de séparation des composants du mélange, distribués sous forme de cercles concentriques, permet d'effectuer un choix. On utilise autant de capillaires qu'il y a de solvants à tester.

Cette microtechnique peut également servir à choisir le système de solvants convenant aux microchromatographies de partage. On utilise souvent, dans ce dernier cas, les solvants mis au point pour des séparations analogues en chromatographie sur papier.

(*e*) *Développement et révélation des microchromatogrammes*

On utilise la technique ascendante. La tendance des taches à la diffusion est inversement proportionnelle à la vitesse du développement et celle-ci, très rapide initialement, diminue fortement après un parcours d'environ 10 cm. Aussi la plupart des auteurs utilisent-ils des distances de développement comprises entre 10 et 20 cm, parcourues en 20 à 120 minutes suivant la viscosité du solvant.

L'emploi de la technique descendante, envisagé par NIEDERWIESER ET PATAKI[14], peut éventuellement contribuer à augmenter les distances de développement utilisables. Un effet plus prononcé dans ce sens pourrait être réalisé par la mise au point d'une méthode centrifuge analogue à celle utilisée en chromatographie sur papier[15].

Il est recommandé de faciliter la saturation de l'atmosphère des cuves de développement, par exemple en recouvrant partiellement leurs parois intérieures de papier-filtre trempant dans le solvant. On améliore ainsi la reproductibilité des valeurs de R_F, supprime l'"effet de bord"[10,25] (augmentation anormale des valeurs de R_F dans les zones marginales des plaques), et augmente la rapidité du développement.

Nous mentionnerons dans les chapitres 2 et 3, sous chaque rubrique spécialisée, les différentes techniques permettant la révélation des microchromatogrammes des substances incolores.

(*f*) *Conservation, mise en archives des microchromatogrammes*

La méthode la plus précise et la plus pratique consiste à photographier les microchromatogrammes en lumière transmise puis à les classer sous forme de diapositives couleur 24 × 36.

La photographie en noir et blanc convient aussi, spécialement en lumières transmise et réfléchie combinées[2], de même que les autres procédés d'usage courant en chromatographie sur papier (photocopie, dessin, etc.).

On peut enfin "fixer" les plaques dans la paraffine[5] et les conserver ainsi.

2. SÉPARATIONS PAR ADSORPTION

(Substances liposolubles)

Il est de règle d'utiliser, pour la séparation des corps liposolubles, des chromatoplaques fortement activées par un chauffage à 100–140° durant 30 à 120 minutes. Les manipulations s'effectuent autant que possible à l'abri de l'humidité atmosphérique.

(*a*) *Mono-, di-, tri- et tétraterpénoïdes*

Dans la classe des terpènes monocycliques, une intéressante étude a été faite par PETROWITZ[16] sur la séparation des menthols. Les épimères dont l'hydroxyle est équatorial (menthol, isomenthol) sont les plus fortement retenus par l'acide silicique. La configuration du groupe méthyle n'exerce de son côté que peu d'effet, d'où l'impossibilité de séparer le menthol de l'isomenthol ou le néomenthol du néoisomenthol. Ces résultats montrent que l'adsorption sur l'acide silicique s'effectue par la fonction hydroxyle, les valeurs de R_F reflétant le degré d'empêchement stérique de celle-ci. On peut rappeler ici que les épimères axiaux des stéroïdes hydroxylés sont généralement aussi les moins adsorbés[17] lorsque le processus chromatographique implique la participation de liaisons hydrogène (chromatographie sur papier).

Le Tableau I résume le comportement des menthols sur chromatoplaque.

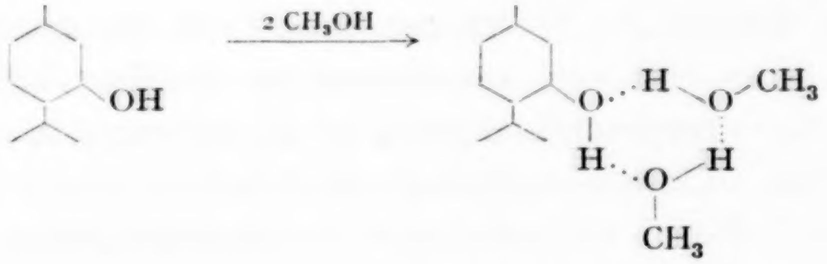
TABLEAU I

CHROMATOGRAPHIE DES MENTHOLS SUR COUCHE SILICIQUE D'APRÈS PETROWITZ[16]

	Valeurs de R_F (× 100)			
	A	*B*	*C*	*D*
Menthol	16	36	67	90
Isomenthol	17	37	62	91
Néomenthol	28	51	73	85
Néoisomenthol	29	55	76	80

Solvants: A = benzène; B = benzène–méthanol (95:5); C = benzène–méthanol (75:25); D = méthanol; trajet de développement: 10–12 cm (50 min); révélation: par l'action de $SbCl_5$ à 20% dans CCl_4.

L'inversion remarquable des valeurs de R_F en présence du méthanol suggère que celui-ci s'associe avec les menthols, particulièrement avec les épimères équatoriaux, les "complexes" ainsi formés devant avoir une adsorbabilité relativement diminuée. On peut par exemple écrire:



Une application de la microchromatographie sur couches minces dans le domaine synthétique a été mentionnée par MARBET ET SAUCY[18]. Ces auteurs parviennent, grâce à cette méthode, à mettre en évidence l'existence d'un cétal acétylénique particulièrement instable, intermédiaire dans une synthèse de la pseudo-ionone.

Dans le domaine des diterpénoïdes, DEMOLE ET LEDERER[19,1] ont décrit la séparation du phytol, de l'isophytol, du géranyl-linalol et de l'acétate de phytyle naturels sur acide silicique. Le développement s'effectue avec le mélange hexane–acétate d'éthyle (85:15, v/v) et la révélation par l'action d'une solution aqueuse de permanganate de potassium à 0.25% (taches brunes sur fond blanc). On constate l'ordre suivant des valeurs de R_F:

acétate de phytyle > isophytol > géranyl-linalol > phytol

Cette séquence suggère que l'adsorption s'effectue principalement par les groupes hydroxyles et secondairement par les doubles liaisons.

Dans la classe des triterpénoïdes, THOMAS ET MÜLLER[20] ont contrôlé sur chromatoplaques siliciques le degré de pureté des esters méthyliques des acides triterpéniques de *Commiphora glandulosa* Schinz. L'éluant est un mélange de chloroforme et d'acétate d'éthyle (4:1) et le révélateur l'acide sulfurique concentré.

TSCHESCHE ET SEN GUPTA[21] ont également utilisé les chromatoplaques pour examiner la pureté des esters de deux nouveaux acides triterpéniques de la série de la β-amyrine présents dans *Bredemeyera floribunda* Willd. Les résultats obtenus, favorables, ont incité TSCHESCHE *et al.*[22] à étudier de plus près les possibilités d'emploi des chromatoplaques siliciques pour l'analyse rapide des triterpénoïdes. Le comportement de 23 acides triterpéniques et de 16 triterpénoïdes neutres (esters, alcools, hydrocarbures) dans 9 systèmes de solvants démontre clairement que la méthode permet d'intéressantes séparations dans ces séries. TSCHESCHE *et al.*[22] soulignent particulièrement les avantages suivants des chromatoplaques:

(1) Le temps nécessaire pour effectuer une séparation est de 20 à 30 minutes.

(2) La méthode convient à la séparation des triterpénoïdes neutres et acides, alors que la chromatographie sur papier n'est habituellement satisfaisante que pour les seconds.

(3) La révélation des triterpénoïdes s'effectue avec une extrême sensibilité (0.02 µg pour l'acide oléanolique) par l'acide chlorosulfonique[6].

(4) Les chromatoplaques peuvent être utilisées pour l'analyse des mélanges très complexes aussi bien que pour des tests de pureté ou l'examen rapide du résultat de réactions chimiques.

Les séparations décrites par TSCHESCHE *et al.*[22] ont été obtenues sur couches de silicagel "G" (Merck) en présence, notamment, de benzène, cyclohexane, chlorure de méthylène (pour les triterpénoïdes neutres) et des mélanges éther di-isopropylique–acétone (5:2), acétate d'éthyle–méthanol–diéthylamine (14:4:3), etc. (pour les triterpénoïdes acides). Parmi les substances chromatographiées nous trouvons les acides oléanolique, ursolique, bétulique, leurs esters, le lanostérol, la β-amyrine, etc. La reproductibilité des valeurs de R_F est satisfaisante si l'on effectue chaque microchromatogramme en présence d'une substance standard (par exemple l'acide oléanolique) permettant la correction des chiffres obtenus.

Le seul insuccès rencontré par TSCHESCHE *et al.*[22] concerne les acides oléanolique, ursolique et bétulique qui possèdent les mêmes valeurs de R_F et doivent être séparés au moyen d'une technique complémentaire spéciale utilisant un papier échangeur d'anions (Schleicher & Schüll).

La séparation de quelques caroténoïdes sur chromatoplaques siliciques en présence du mélange hexane–éther diéthylique (3:7, v/v) a été décrite par DEMOLE[1]. Valeurs de R_F: β-carotène 0.96; canthaxanthine 0.38; zéaxanthine 0.17; isozéaxanthine 0.63; bixine 0.51.

ISLER *et al.*[23] ont d'autre part décrit le comportement de β-apocaroténals (C_{25}–C_{40}) et β-apocaroténoates de méthyle (C_{27}–C_{30}) sur un adsorbant spécial (mélange $Ca(OH)_2$–silicagel "G" (Merck), 6:1).

STAHL[10,24] a également mentionné l'usage de chromatoplaques pour la séparation de caroténoïdes.

(b) *Stéroïdes*

La microchromatographie d'acides biliaires et d'autres stéroïdes a été mentionnée par STAHL[24,25] avec peu de détails expérimentaux.

BARBIER *et al.*[3] ont utilisé les chromatoplaques siliciques pour l'analyse de stéroïdes peu polaires et obtenu des séparations qui n'avaient pu être réussies sur papier. Leur technique, classique, utilise le silicagel "G" (Merck) et des mélanges d'acétate d'éthyle et de cyclohexane en proportions variables comme solvants. Après deux heures de développement (parcours du front du solvant de 175 mm environ), les chromatogrammes sont révélés par $SbCl_3$ en solution chloroformique, par l'acide phosphomolybdique ou, dans le cas des cétones stéroïdes, par une solution acide de 2,4-dinitrophénylhydrazine. BARBIER *et al.*[3] donnent les valeurs de R_F de 22 stéroïdes peu polaires (il s'agit principalement de cétones, esters, alcools et époxydes 3β- et 3α-acétoxylés). Comme pour les triterpénoïdes vus précédemment, les séparations doivent être effectuées en présence d'un témoin permettant la correction des valeurs de R_F. Les chiffres obtenus sont constants de ± 0.05 à ± 0.1 près suivant les cas.

Dans le cas des stéroïdes polaires (comme pour les substances polaires en général) on obtient de meilleurs résultats en utilisant des chromatoplaques partiellement désactivées par un séjour à l'air libre. On se rapproche ainsi des conditions d'une chromatographie de partage, l'eau adsorbée fonctionnant comme phase stationnaire. BARBIER *et al.*[3] ont appliqué ce principe à la séparation des acides cholique (R_F = 0.32) et désoxycholique (R_F = 0.62) en présence d'acétate d'éthyle.

On peut identifier certains stéroïdes en observant, sous lumière ordinaire ou ultra-violette, le nuancement plus ou moins spécifique de la réaction colorée produite par le $SbCl_3$. Ceci permet aussi la différenciation de stéroïdes qui possèdent des valeurs de R_F presque identiques.

VAN DAM *et al.*[26] ont mis au point la séparation du cholestérol, d'esters de cholestérol, de cholestènediols et de quelques 3β-acétoxy-cétones stéroïdes. Leur technique permet d'examiner rapidement et commodément les produits complexes résultant de la dégradation oxydative de la chaîne latérale du cholestérol. En 90 minutes, certains mélanges ont été fractionnés en plus de 20 composants, dont plusieurs identifiés avec certitude. Les chromatoplaques sont à base de silicagel, le développement étant assuré par des mélanges de benzène, toluène, cyclohexane et acétate d'éthyle. Après un parcours du front du solvant d'environ 18 cm (40 à 100 minutes), les stéroïdes sont révélés par $SbCl_3$ (taches de couleurs variées). Les valeurs de R_F de 17 d'entre eux ont été déterminées dans 13 solvants.

Le comportement de 13 cardénolides et dérivés sur chromatoplaques siliciques a été décrit par TSCHESCHE *et al.*[6]. Après 10 à 30 minutes de développement (dans l'acétate d'éthyle ou l'acétate de butyle), les plaques subissent une révélation au moyen du mélange acide acétique–acide chlorosulfonique (2:1). Cette réaction, particulièrement sensible, donne des taches vertes à fluorescence brun-violet dans l'ultra-violet.

TSCHESCHE ET SNATZKE[27] ont encore utilisé les chromatoplaques pour séparer 13 autres stéroïdes (cholestérol, β-sitostérol, 5α-prégnanedione-3,20, Δ^4-prégnènetrione-3,6,20, etc.). Le développement s'effectue avec des solvants moins polaires que dans le cas des cardénolides (éther di-isopropylique, mélange éther di-isopropylique–acétate d'éthyle (5:2), benzène). Les auteurs soulignent une nouvelle fois la remarquable sensibilité de la révélation par l'acide chlorosulfonique[6] qui leur permet de déceler jusqu'à 0.025 μg de cholestérol sur un microchromatogramme développé (en série triterpénique, le seuil de sensibilité est de 0.02 μg pour l'acide oléanolique).

Pour terminer, mentionnons encore un travail de BARBIER ET ZAV'YALOV[28] concernant la séparation des stéroïdes oestrogènes sur chromatoplaques.

(*c*) *Huiles essentielles, peroxydes*

STAHL[2,29] a examiné certaines huiles essentielles sur chromatoplaques siliciques avec, généralement, le benzène comme solvant. Pour révéler les microchromatogrammes, il recourt à l'action de $SbCl_3$ ou mieux de $SbCl_5$ (réactif universel pour les terpènes), au test brome–fluorescéine[30] (pour les substances insaturées) et à l'effet de masquage de fluorescence[30] (pour les substances absorbant suffisamment dans l'ultra-violet).

Dans le cas des huiles essentielles contenant des peroxydes, ceux-ci peuvent être détectés au moyen de tests au sulfocyanure ferrique ou à l'iodure de potassium[31,32].

WINKLER ET LUNAU[33] ont différencié sur chromatoplaque les huiles essentielles de *Curcuma xanthorrhiza* et *Curcuma longa*.

PRYOR ET BRYANT[7] ont étudié la composition d'huiles essentielles d'espèces hybrides d'*Eucalyptus* au moyen de la chromatographie radiale sur magnésol (silicate hydraté de magnésium; l'acide silicique donnerait de moins bons résultats dans ce cas particulier). Après développement (30 minutes) avec le système hexane–acétate d'éthyle (85:15, w/w), les chromatogrammes circulaires sont révélés par l'acide sulfurique concentré ou examinés en lumière ultra-violette. A l'exception du *p*-cymène et des pinènes, la plupart des composants des essences d'*Eucalyptus* donnent des réactions caractéristiques avec l'acide sulfurique.

On doit à STAHL ET TRENNHEUSER[34] une démonstration de l'intérêt que peut présenter, notamment pour l'étude d'huiles essentielles, l'utilisation combinée de la chromatographie gaz–liquide et de la microchromatographie sur couches minces.

FRYDMAN *et al.*[35] ont également utilisé les chromatoplaques pour l'examen direct et systématique d'huiles essentielles.

(d) *Pyréthrines, coumarines, mono- et polyphénols, quinones*

STAHL[36] a mis à contribution les avantages de la microchromatographie sur couches minces pour étudier le réarrangement des pyréthrines en lumi-pyréthrines inactives sous l'effet de la lumière. Sa technique, dite "séparation–réaction–séparation", est inspirée des tests microchimiques sur "chromatostrips" décrits par MILLER ET KIRCHNER[37]. Elle consiste à soumettre un microchromatogramme unidimensionnel à l'action de certains réactifs (gaz, rayonnements), puis à le développer dans la seconde dimension afin de mettre en évidence les nouveaux produits éventuellement formés.

BERNHARD[38] a détecté au moyen de "chromatostrips" la présence, dans le jus de citron, de huit analogues fluorescents de la coumarine et proposé une identité (basée sur les valeurs de R_F et les spectres d'absorption ultra-violette) pour cinq d'entre eux.

La même technique a permis à LYMAN *et al.*[39] d'étudier la méthylation séquentielle de l'acide *β*-résorcylique. L'examen du produit en cours de méthylation, sur "chromatostrips" fluorescents, permet de suivre et d'interrompre judicieusement la réaction afin d'obtenir le dérivé désiré. La méthylation de l'acide *β*-résorcylique en milieu alcalin livre d'abord l'ester correspondant, ensuite le dérivé méthylé en 4 puis diméthylé en 2,4. Dans cette série, le solvant utilisé est un mélange éther diéthylique–skellysolve B* (7:3). LYMAN *et al.*[39] ont donné les valeurs de R_F de 26 mono- et polyphénols déterminées dans trois systèmes de solvants et indiqué les couleurs de fluorescence de ces substances à 254 mμ.

STAHL[10] a également mentionné la microchromatographie de quelques phénols et dérivés (eugénol, pyrocatéchine et éthers correspondants, safrol).

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* Éther de pétrole bouillant à 66–67°.

BARBIER[40] a obtenu une excellente séparation des *p*-benzoquinones substituées, sur couches siliciques désactivées, en présence de mélanges de benzène ou d'hexane avec l'acétate d'éthyle. Cet auteur recommande l'usage des chromatoplaques pour la recherche préliminaire des *p*-benzoquinones naturelles et indique les valeurs de R_F suivantes (*n*-hexane–acétate d'éthyle, 85:15): *p*-benzoquinone 0.21; méthyl-*p*-benzoquinone 0.43; éthyl-*p*-benzoquinone 0.58; méthyl-2 diméthoxy-5,6 *p*-benzoquinone 0.47; dihydroxy-2,5 *n*-undécyl-3 *p*-benzoquinone 0.62.

(*e*) *Dérivés d'acides aminés, alcaloïdes et autres substances azotées*

Les phénylthiohydantoïnes des acides aminés N-terminaux, résultant de la dégradation des peptides d'après EDMAN, peuvent être identifiées sur chromatoplaques siliciques. CHERBULIEZ *et al.*[41] ont donné des exemples de séparations en soulignant la sensibilité remarquable de la méthode. On peut en effet obtenir de bons résultats à partir de 0.01 μmole de di- ou tripeptides, ce seuil étant 10 à 20 fois plus élevé en chromatographie sur papier. Le solvant utilisé pour séparer les phénylthiohydantoïnes de la glycine, de la proline et de la leucine, est un mélange heptane–pyridine–acétate d'éthyle (5:3:2, v/v). La révélation des chromatogrammes (développés en 30 minutes environ) s'effectue par pulvérisation d'une solution aqueuse d'amidon suivie d'un réactif à l'iode et azoture de sodium. Les phénylthiohydantoïnes apparaissent en blanc sur fond brun.

SCHLEMMER ET LINK[42] ont caractérisé la réserpine, la rescinnamine et la serpentine par microchromatographie sur silicagel de divers extraits de *Rauwolfia*. Ces alcaloïdes sont détectés en lumière ultra-violette et dosés spectrophotométriquement après micro-élution des taches (découpées au rasoir).

FIORI ET MARIGO[43] ont identifié le méprobamate (drogue tranquillisante) dans les urines par microchromatographie silicique en présence du mélange cyclohexane–éthanol absolu (85:15). Après un développement de deux heures (parcours du front du solvant: 10 à 11 cm), les chromatoplaques subissent une légère pulvérisation d'acide sulfurique concentré, un chauffage de 2 à 3 minutes à 110–115°, une nouvelle légère pulvérisation d'eau et un nouveau chauffage de plusieurs minutes, jusqu'à l'apparition des taches jaunes du méprobamate (R_F = 0.30). Des déterminations colorimétriques peuvent être effectuées après découpage et élution des taches.

MACHATA[4] a introduit les chromatoplaques dans le domaine de l'analyse toxicologique où leur emploi semble prometteur par rapport à la chromatographie classique sur papier (économie de temps, simplicité de la méthode et faculté d'effectuer aisément des séparations à l'échelle de quelques milligrammes). Cet auteur décrit le comportement chromatographique d'un certain nombre d'alcaloïdes (papavérine, narcotine, codéine, morphine, etc.) et d'autres substances intéressant le domaine toxicologique. Il développe les chromatogrammes des substances basiques avec le méthanol et acides avec le mélange chloroforme–éther diéthylique (85:15). La révélation peut s'effectuer avec le réactif de Dragendorff ou le mélange acide acétique–iode–iodure de potassium pour les alcaloïdes, par le réactif de Zwikkers pour les barbiturates, le chlorure ferrique en solution acétique pour les pyrazolones etc. Les alcaloïdes de l'opium peuvent de plus être différenciés par l'action du réactif de Marquis, trop corrosif pour l'usage en chromatographie sur papier. Dans les cas où il n'existe aucune méthode particulière de révélation, on chauffe rapidement les chromatoplaques à 500° et observe les taches de carbonisation.

DEMOLE[1] a chromatographié certains pigments tétrapyrroliques sur acide silicique en présence du mélange benzène–acétate d'éthyle–éthanol (90:20:7.5, v/v) et observé les valeurs de R_F suivantes: coproporphyrine I (ester méthylique) 0.68; coproporphyrine III (e.m.) 0.63; uroporphyrine I (e.m.) 0.33; deutéroporphyrine (e.m.) 0.80; protoporphyrine (e.m.) 0.85; bilirubine 0.92; biliverdine 0.06.

La séparation d'autres substances azotées a été mentionnée par STAHL[10] (alcaloïdes de l'ergot de seigle, diphénylamine, carbazol, indol).

Pour l'analyse de vitamines du groupe B, voir le paragraphe suivant.

(f) *Vitamines*

L'adaptation des chromatoplaques à la séparation de vitamines du groupe B (B_1, B_2, B_6, nicotinamide, biotine, pantothénate de calcium) et de la vitamine C a été réalisée par GÄNSHIRT ET MALZACHER[11]. La microchromatographie s'effectue sur une couche de silicagel "G" (Merck) contenant 2% d'un indicateur fluorescent* en présence du mélange acide acétique–acétone–méthanol–benzène (5:5:20:70). Le développement, unidimensionnel (distance de migration: 19 cm), est réalisé à l'abri de la lumière. Chaque mélange examiné doit être chromatographié à double (deux taches de départ) afin de permettre l'exécution des trois réactions de révélation décrites ci-dessous.

On examine tout d'abord les microchromatogrammes sous éclairage ultra-violet de grande longueur d'onde (366 mμ), qui ne provoque pas la fluorescence de l'indicateur incorporé. La vitamine B_2 ($R_F = 0.35$) apparaît sous forme d'une tache fluorescente jaune sur un fond sombre. Passant à l'éclairage ultra-violet de courte longueur d'onde (254 mμ) on détecte, sur les plaques devenues fluorescentes, les taches sombres correspondant aux vitamines B_1 ($R_F = 0$), C ($R_F = 0.30$) et à la nicotinamide ($R_F = 0.65$). Pour identifier la biotine, on pulvérise une solution d'iodo-platinate de potassium sur l'un des microchromatogrammes (l'autre étant protégé par un cache). La biotine ($R_F = 0.80$) forme alors une tache blanche sur fond rose (simultanément, la vitamine B_1 apparaît en gris, la nicotinamide en jaune pâle et la vitamine C en jaune). Pour rechercher la vitamine B_6 et le pantothénate de calcium, on pulvérise sur la partie inférieure du second microchromatogramme (jusqu'alors protégé par le cache) une solution à 0.1% de dichloroquinone-chloroimide dans l'éthanol puis expose aux vapeurs ammoniacales. La vitamine B_6 ($R_F = 0.15$) apparaît en bleu. On chauffe ensuite la plaque 1/2 heure à 160° puis pulvérise une solution de ninhydrine à 0.5% dans l'éthanol sur la partie supérieure du même chromatogramme. Après un bref chauffage à 160°, le pantothénate de calcium ($R_F = 0.57$) apparaît sous forme de tache violette.

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* "ZS-Super" de Riedel de Haen.

Sensibilité de la méthode: 1 à 10 μg pour les vitamines du groupe B et 5 à 30 μg pour la vitamine C.

(*g*) *Antioxydants, plastifiants*

SEHER[45] a trouvé que l'usage des chromatoplaques simplifie la recherche et l'identification des antioxydants. Il pulvérise uniformément, sur les microchromatogrammes développés, une solution méthanolique à 20% d'acide phosphomolybdique puis, après une ou deux minutes, expose les plaques au contact de vapeurs ammoniacales. Les antioxydants apparaissent sous forme de taches bleues, violettes ou vertes sur fond blanc. Pour révéler les antioxydants peu réactifs, il peut être nécessaire de chauffer les plaques à 120° durant 10 minutes. Une autre méthode de révélation consiste en une aspersion de 2,6-dichloroquinone-chloroimide (en solution alcoolique) suivie de borax (dans le même solvant). Les colorations produites sont caractéristiques et l'auteur les décrit pour 24 antioxydants.

COPIUS PEEREBOOM[46] a utilisé les chromatoplaques pour le contrôle des plastifiants présents dans certains emballages pour denrées alimentaires. Les séparations se font sur silicagel en présence de 0.005% d'un indicateur fluorescent*. Les microchromatogrammes, développés avec l'un des mélanges isooctane–acétate d'éthyle (9:1), benzène–acétate d'éthyle (95:5) ou éther dibutylique–hexane (80:20), sont examinés en lumière ultra-violette (365 mμ). Les plastifiants apparaissent sous forme de taches fluorescentes ou, dans le cas des phtalates, de taches sombres. La reproductibilité des valeurs de R_A ($R_A = R_F$ par rapport au sébaçate de dibutyle) paraît satisfaisante pour les 16 produits étudiés (limites de fluctuation ± 0.06). Certains réactifs donnant des réactions colorées plus ou moins spécifiques peuvent faciliter l'identification des plastifiants, surtout dans le cas de trois "paires critiques" de valeurs de R_F très voisines (phosphate de tricrésyle + butyl-phtalyl-butyl glycolate, phosphate de tricrésyle + phosphate de 2-éthylhexyle diphényle; ce dernier + acétylcitrate de tributyle). Parmi ces réactifs, on peut mentionner l'acide phosphomolybdique, la 2,6-dichloroquinone-chloroimide, le permanganate de potassium, la *p*-nitroaniline diazotée, etc. Les valeurs de R_A et les réactions colorées permettent d'identifier, dans la plupart des cas, les plastifiants d'un mélange inconnu. Toutefois, certains de ceux-ci, par exemple le Paraflex G62, de composition complexe, donnent une série de taches pouvant entraver sérieusement l'identification des autres substances éventuellement présentes.

(*h*) *Divers*

STAHL[24,25] a parfaitement bien démontré l'intérêt que présente la microchromatographie sur couches minces dans les domaines pharmaceutique (contrôles de résines, baumes et goudrons médicinaux) et cosmétologique (contrôles de matières premières: huiles essentielles, parfums, baumes, résinoïdes, graisse de laine et dérivés, colorants liposolubles, etc.).

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* Par exemple "Ultraphor", Badische Anilin & Soda Fabrik.

Les chromatoplaques peuvent également permettre le contrôle d'excipients pour médicaments[47] et l'étude de lipides[48].

3. SÉPARATIONS PAR PARTAGE

(Substances hydrosolubles)

(a) *Généralités*

La microchromatographie de partage sur couches minces s'effectue le plus souvent sur silicagel désactivé et en présence de solvants aqueux.

Niederwieser et Pataki[14] ont fort bien défini les avantages présentés par cette technique pour la séparation des acides aminés, peptides et dérivés. Comparant un chromatogramme bi-dimensionnel d'acides aminés sur papier avec le même (solvants et dimensions identiques) effectué sur silicagel, ces auteurs soulignent:

1. La diffusibilité relativement réduite des substances dans la couche silicique. Les taches obtenues étant très petites, les séparations sont meilleures et la méthode devient dix fois plus sensible que la chromatographie sur papier.

2. L'obtention d'une séparation incomplète de dix acides aminés sur papier (après un développement de dix centimètres dans chacune des dimensions), alors que quatorze de ceux-ci se séparent complètement sur acide silicique.

Solvants: (a) *n*-butanol–acide acétique–eau (60:20:20); (b) phénol–eau (75:25, poids).

3. La durée du développement pour une dimension, réduite à 1 1/2–2 h sur chromatoplaque.

Les chromatoplaques siliciques possèdent de plus une excellente sélectivité. La séparation de peptides isomères, par exemple glycyl-leucine et leucyl-glycine, s'effectue facilement.

De bons résultats peuvent aussi être obtenus là où la chromatographie sur papier réussit difficilement, notamment dans le cas des substances à groupe–SH libre (dérivés de la cystine). Les chromatoplaques permettent en outre la séparation des dérivés dinitrophénylés d'acides aminés et par conséquent l'analyse séquentielle des peptides. La micro-élution des taches s'effectue en découpant l'adsorbant à l'endroit voulu, épuisant avec un solvant convenable, puis filtrant. On ne peut utiliser l'eau pour cette opération lorsque les couches siliciques sont "liées" avec du plâtre (légèrement soluble). On doit alors utiliser, par exemple, le *n*-butanol ou l'acide acétique.

La révélation s'effectue à l'aide de réactifs corrosifs (acide sulfurique concentré, etc.) aussi bien qu'avec ceux habituellement utilisés en chromatographie sur papier (ninhydrine, etc.). Les taches colorées pâlissent souvent assez rapidement et ceci oblige à prendre immédiatement une copie des microchromatogrammes.

En lumière ultra-violette, les couches siliciques (silicagel "G", Merck) présentent un "fond" d'absorption qui gêne la détection des petites quantités d'acide aminés aromatiques. On peut partiellement remédier à cet inconvénient en pulvérisant une solution de fluorescéine à $10^{-4}\%$ sur les microchromatogrammes. L'usage d'adsorbants autres que le silicagel "G" (Merck) permettrait éventuellement de supprimer les désavantages inhérents à ce "fond" d'absorption et à la présence de plâtre.

La reproductibilité des valeurs de R_F (technique ascendante) dépend beaucoup du degré de saturation de l'atmosphère de la cuve de développement; aussi recommande-t-on de disposer contre les parois de celle-ci des feuilles de papier-filtre trempant dans la phase mobile. L'emploi d'une technique de développement descendante, proposé par NIEDERWIESER ET PATAKI[14], pourrait éliminer ce genre de difficulté.

(b) Acides aminés

MUTSCHLER ET ROCHELMEYER[49] ont effectué la chromatographie bi-dimensionnelle de 14 acides aminés sur silicagel en présence d'un tampon phosphate, avec de l'éthanol à 70% et le mélange éthanol–ammoniaque à 25% (4:1, v/v) comme solvants. La très belle séparation obtenue a été confirmée par BRENNER ET NIEDERWIESER[50] qui ont étudié avec un soin particulier l'applicabilité des chromatoplaques à l'analyse des acides aminés.

Ces derniers auteurs constatent que, parmi les solvants habituellement utilisés en chromatographie sur papier, ceux riches en méthanol, éthanol ou acétone permettent souvent de bonnes séparations sur chromatoplaques mais donnent des taches relativement diffuses. On obtient, de loin, les meilleurs résultats par chromatographie bi-dimensionnelle avec les systèmes *n*-butanol–acide acétique–eau (60:20:20) et phénol–eau (75:25, poids).

Les couches siliciques utilisées pour la séparation des acides aminés ne doivent pas être activées à chaud, mais simplement séchées à la température ambiante et à l'air libre. On dépose les acides aminés (1 à 5 µg) à 0.8 cm les uns des autres sur une ligne située à 1.5 cm de la base des chromatoplaques (soit 20 substances par plaque de 20 × 20 cm). Cette opération peut être effectuée à l'aide d'une micro-pipette ou de capillaires étirés par soi-même, la règle essentielle étant de ne pas déposer plus de 1 mm^3 de solution à la fois afin que les taches initiales restent suffisamment petites. On stoppe habituellement le développement du microchromatogramme après un parcours du front du solvant d'environ 10 cm. Il n'y a guère d'avantage à utiliser de plus grands trajets, ceux-ci favorisant la diffusion des taches. Il est recommandé de couper l'adsorbant d'un trait de crayon, à 10 cm au-dessus de la ligne de départ, et d'interrompre le chromatogramme au moment exact où le solvant atteint ce repère. Les valeurs de R_F obtenues de la sorte sont reproductibles à ± 0.05 près (Tableau II). Si la cuve contient plusieurs plaques, on ne trace de trait que sur la première, puis, son développement étant terminé, on note le niveau atteint par le solvant sur chacune des autres. Ce mode opératoire s'impose car le niveau atteint par le solvant à la fin du développement peut différer légèrement d'une plaque à l'autre.

Les valeurs de R_F fluctuent, dans des limites ordinairement négligeables, suivant la quantité de substance chromatographiée. La grosseur des taches dépend bien entendu de ce même facteur et leur surface, dans une zone de concentrations moyennes (1 à 20 µg environ), est proportionnelle au log de la quantité de substance, comme en chromatographie sur papier.

Pour révéler les microchromatogrammes, on les sèche à 110° durant 10 minutes

TABLEAU II

CHROMATOGRAPHIE DES ACIDES AMINÉS SUR COUCHE SILICIQUE
D'APRÈS BRENNER ET NIEDERWIESER[50]

	R_F (× 100)			R_F (× 100)	
	A	*B*		*A*	*B*
Tryptophane	56	63	Thréonine	25	26
Phénylalanine	49	55	Sérine	22	20
Norleucine	49	52	Glycine	22	24
Leucine	47	48	Hydroxy-proline	20	38
Isoleucine	46	49	Proline	19	50
Tyrosine	47	47	Sarcosine	17	37
Dioxyphénylalanine	45	34	Ac. glutamique	27	10
Méthionine	40	49	Ac. aspartique	21	06
Norvaline	38	42	Histidine-HCl	06	32
Valine	35	40	Lysine-HCl	05	09
Alanine	27	29	Arginine-HCl	08	19
β-Alanine	27	30	Cystine-HCl	16	12

Solvants: A = *n*-butanol–acide acétique–eau (60:20:20); B = phénol–eau (75:25, poids).

puis les soumet à une pulvérisation soigneuse du réactif à la ninhydrine modifié par MOFFAT ET LYTLE[51]. On chauffe à nouveau en suivant, par transparence, l'apparition graduelle des taches colorées. Il est souvent possible de différencier des acides aminés migrant presque ensemble, s'ils se révèlent initialement sous forme de taches extrêmement petites ne s'élargissant que progressivement. Les acides aminés se distinguent en outre par la plus ou moins grande rapidité de leur réaction avec la ninhydrine. Malgré ce comportement spécifique, les composants de certains mélanges ne peuvent être tous différenciés à coup sûr (par exemple: Phe + Leu + Ileu et Ser + Hypro + Gly).

L'avantage primordial de la chromatographie de partage des acides aminés sur couches minces réside dans l'économie de temps réalisée (4 à 5 heures pour un chromatogramme bi-dimensionnel contre 2 à 3 jours en chromatographie sur papier).

MOTTIER[5] a étudié le comportement chromatographique d'acides aminés et d'hydrolysats protéiques sur plaque d'alumine. Sa technique, appliquée précédemment à l'analyse de colorants alimentaires, est caractérisée par le fait que les couches adsorbantes ne contiennent pas d'agent liant. L'alumine utilisée doit répondre à des critères de qualité et d'activation bien définis. Les acides aminés sont chromatographiés à l'état de sels de sodium (alcalinisation des taches de départ) en présence de 4 systèmes de solvants. Le meilleur de ceux-ci, le mélange *n*-butanol–éthanol–eau (60:40:40, v/v), conduit aux résultats résumés dans le Tableau III, la durée du développement étant de 8 heures.

Du fait de l'absence d'agent liant, les couches adsorbantes préparées d'après MOTTIER[5] sont fragiles. On peut se demander si des chromatoplaques conventionnelles, évidemment moins délicates, ne pourraient les remplacer avec profit.

TABLEAU III

CHROMATOGRAPHIE DES ACIDES AMINÉS SUR COUCHE ALUMINIQUE, D'APRÈS MOTTIER[5]

	R_F (× 100)*		R_F (× 100)*
Tryptophane	100	Hydroxy-proline	43.5
Proline	87	Histidine	39
Phénylalanine	87	Glycine	35
Norleucine	79	Thréonine	34
Isoleucine	75	Sérine	32.5
Leucine	71	Arginine	29
Norvaline	62	Ac. glutamique	29
Tyrosine	56.5	Cystine	29
Valine	55.5	Lysine	28
Méthionine	51	Cystéine	20.5
Alanine	47	Ac. aspartique	19.5
Carnosine	44.5		

* Valeurs de R_F relatives au tryptophane.
Solvant: *n*-butanol–éthanol–eau (60:40:40, v/v).
Note: il est intéressant de comparer ces chiffres à ceux du Tableau II.

(*c*) *Ptéridines*

NICOLAUS[52] a utilisé les chromatoplaques siliciques pour l'examen de la pureté et la séparation micro-analytique de ptéridines. Sa méthode supporte avantageusement la comparaison avec la chromatographie classique sur papier. Certaines ptéridines, difficilement séparables au moyen de cette dernière, se laissent parfaitement chromatographier sur chromatoplaques.

Vingt-quatre systèmes de solvants (neutres, acides et basiques) ont été étudiés pour la séparation de 12 ptéridines. La chromatographie des amino-2 hydroxy-4 ptéridines à caractère neutre peut s'effectuer avec l'un quelconque de ces types de solvants, les meilleurs résultats étant cependant obtenus en milieu acide. Le mélange *n*-butanol–acide acétique–eau donne de bons résultats avec les ptéridines méthylées en position 6 ou 7. Dans le cas des amino-2 hydroxy-4 ptéridines portant des substituants acides, on utilise de préférence un milieu basique. La dihydroxy-2,4 ptéridine migre tout aussi bien en milieu neutre, acide ou basique.

Le développement des microchromatogrammes nécessite en moyenne 20 minutes (parcours du front du solvant: environ 10 cm) et la quantité nécessaire de ptéridines varie de 1 à 10 μg. L'identification est assurée par les valeurs relatives de R_F et par l'observation des couleurs de fluorescence dans l'ultra-violet, partiellement caractéristiques. Ce même nuancement dans la fluorescence permet la différenciation de ptéridines migrant presque ensemble.

L'oxyde d'aluminium ne semble guère convenir pour la séparation de ce genre de substances (adsorption trop forte).

(*d*) *Ions minéraux*

SEILER ET SEILER[53] ont pu adapter la chromatographie sur couches minces à la séparation de cations, non sans s'être heurtés à certaines difficultés préalables. Par exemple, le silicagel "G" (Merck) contient du fer, fort gênant, et doit être soumis à une purification spéciale (lavages acides). D'autre part, les couches adsorbantes se dissociant au contact des solvants contenant trop d'eau, ceci oblige à effectuer les développements en milieu relativement peu polaire.

SEILER ET SEILER[53] préparent leurs chromatoplaques d'une manière conventionnelle, sans les activer à chaud. La séparation des cations du groupe H_2S (résultant du fractionnement analytique classique) s'effectue en présence du mélange *n*-butanol–HCl 1.5 *N*–acétonylacétone (100:20:0.5, v/v), moyennant un développement de deux heures (12 à 14 heures en chromatographie sur papier). Celle du groupe $(NH_4)_2S$ s'effectue avec le mélange acétone–HCl concentré–acétonylacétone (100:1:0.5, v/v), en 15 à 20 minutes (10 à 12 heures sur papier). Le rôle de l'acétonylacétone (agent complexant) est de diminuer la "traînée" des taches.

Les valeurs de R_F absolues étant moins constantes qu'en chromatographie sur papier, on identifie les différents cations d'après leur ordre de distribution sur les chromatogrammes et leurs réactions colorées spécifiques.

La révélation des cations du groupe H_2S s'effectue en pulvérisant une solution de KI à 2% sur le chromatogramme. Après séchage, on porte la plaque au contact de vapeurs ammoniacales puis de H_2S. On observe les colorations indiquées dans le Tableau IV.

TABLEAU IV

Cations	*Réactions caractéristiques*	
	KI	*H_2S*
Hg^{2+}	rouge	brun-noir
Bi^{3+}	brun-jaune	brun-noir
Cd^{2+}		jaune
Pb^{2+}	jaune-brun	brun
Cu^{2+}	brun	brun foncé

Les valeurs de R_F décroissent de Hg^{2+} à Cu^{2+}.

Pour révéler le groupe $(NH_4)_2S$, on plonge tout d'abord les chromatogrammes dans une atmosphère d'ammoniac puis les soumet à une pulvérisation d'une solution de 0.5 g d'hydroxy-8 quinoléine dans 100 cm³ d'éthanol à 60%. On termine par l'observation des taches en lumière ultra-violette (Tableau V).

TABLEAU V

Cations	*Réactions caractéristiques*		
	NH_3	*Oxine*	*U.V.*
Fe^{3+}		brun	foncé
Zn^{2+}		rose	jaune
Co^{2+}	bleu	jaune	foncé
Mn^{2+}		orangé	foncé
Cr^{3+}	vert		foncé
Ni^{2+}			foncé
Al^{3+}			jaune clair

Les valeurs de R_F décroissent de Fe^{3+} à Al^{3+}.

4. CONCLUSIONS

Dans cette seconde revue, nous avons relaté les progrès essentiels réalisés par la microchromatographie sur couches minces entre 1958 et 1960. Cette méthode, introduite en 1951 par Kirchner *et al.*[30], perfectionnée par Reitsema[54], normalisée par Stahl[2], se révèle aujourd'hui comme exceptionnellement universelle dans ses applications. Elle permet de séparer des substances indifféremment lipo- ou hydrosolubles, présente l'avantage d'une grande rapidité alliée à une excellente sélectivité et à une sensibilité souvent supérieure à celle de la chromatographie sur papier. Le matériel nécessaire en pratique, simple et peu coûteux, est actuellement commercialisé. On ne saurait trouver un indice plus sûr de l'intérêt général soulevé par cette méthode.

Le domaine d'application de la microchromatographie sur couches minces va probablement s'agrandir encore ces prochaines années, notamment en ce qui concerne la séparation des substances hydrosolubles (acides aminés, sucres et dérivés, etc.). On peut souhaiter que l'usage intensif de cette méthode stimulera l'étude théorique des relations existant entre le comportement chromatographique et la structure moléculaire des substances.

REMERCIEMENTS

L'auteur exprime sa vive reconnaissance au Dr. M. Stoll, Directeur scientifique, et à la Maison Firmenich & Cie, Genève, pour leur appui et approbation.

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NOTE AJOUTÉE DURANT LA CORRECTION DES ÉPREUVES

Les références supplémentaires suivantes, venant de parvenir à notre connaissance, doivent encore être mentionnées:

(a) Analyse de mélanges complexes de lipides par chromatographie sur couches minces et méthodes complémentaires

D. C. Malins et H. K. Mangold, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576; *cf. Current Chem. Papers*, No. 1 (1961) 60.

(b) Séparation de lipides

H. P. Kaufmann et Z. Makus, *Fette, Seifen, Anstrichmittel*, 62 (1960) 1014.

(c) Analyse de mélanges de tocophérols à l'aide de la chromatographie sur couches minces

A. Seher, *Mikrochim. Acta*, (1961) 308.

(d) Analyse d'esters p-hydroxy-benzoïques

H. Gänshirt et K. Morianz, *Arch. Pharm.*, 293 (1960) 1065.

(e) Séparation d'amines

K. Teicher, E. Mutschler et H. Rochelmeyer, *Deut. Apotheker-Ztg.*, 100 (1960) 283.

(f) Ionophorèse et chromatographie-ionophorèse sur couches minces (acides aminés et amines)

C. G. Honegger, *Helv. Chim. Acta*, 44 (1961) 173.

(g) Séparation de peptides et dérivés sur couches minces

R. L. Huguenin et R. A. Boissonnas, *Helv. Chim. Acta*, 44 (1961) 219.

LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

II. COMPOUND SEPARABILITY WITH ALUMINA AS ADSORBENT

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(Received September 20th, 1960)

INTRODUCTION

The theoretical possibility of linear elution adsorption chromatography (LEAC) in the case of adsorbents which are energetically heterogeneous has been discussed, and experimental examples of the phenomenon have been cited for chromatographic systems featuring both activated alumina and silica as adsorbents[1]. In the related area of liquid–liquid and gas–liquid elution chromatography, PIEROTTI *et al.*[2] and REDLICH *et al.*[3] have used a molecular building block approach to relate solute structure to equilibrium solute activity coefficients at infinite dilution; these coefficients are in turn directly relatable to linear elution retention volumes in the corresponding chromatographic systems. Similarly, REICHL[4] has shown that R_M values in certain paper chromatographic systems may be accurately predicted as the sum of structural group parameters. SPORER AND TRUEBLOOD[5] have made use of the same model in the correlation of a number of compound separation factors for the chromatographic system benzene–silica. These latter authors have postulated a simple additive free energy relationship for the adsorption equilibrium involving a solute with multiple functional groups, while recognizing certain theoretical limitations in this model. A considerable number of other published studies[6–8] have treated the question of compound chromatographic separability as it relates to molecular structure; few such studies have been carried out for chromatographic systems known to be linear. The general desirability of limiting experimental data of this type to linear systems has been discussed previously[1,5], especially where the quantitative interpretation of adsorption phenomena is important.

The use of different eluents in these chromatographic studies, as well as adsorbents that have been water-deactivated to varying extents, further complicates even the qualitative comparison of the results of different workers. The need for a general theoretical model which is capable of interrelating solute chromatographic separability, solute molecular structure, adsorbent deactivation and eluent structure is apparent. Given such a correlational scheme, it should be possible to predict, at least semi-quantitatively, separation factors for a large number of experimental LEAC systems (and to estimate separation order in non-linear systems) on the basis of a limited number of observations and a small number of theoretical concepts. Of equal importance, where the theoretical model is incomplete or inaccurate, comparison of calculated and experimental data can facilitate the recognition of defects in the existent science of adsorption chromatography.

EXPERIMENTAL

All of the chromatographic data described in the present and previous[1] communications were obtained by the following general procedure. The glass and teflon apparatus of Fig. 1 was used, with the various ball and socket joints externally coated with a plastic paint in earlier runs. Later studies dispensed with this feature, since it was found that leakage was invariably small and confined to joints between the eluent reservoir and the beginning of the chromatographic column; this had no effect on the data obtained. Columns (10 mm × 50–500 mm) were packed dry to maximum density by tamping and vibrating, the adsorbent weight determined by difference, the columns mounted, and then wetted with solvent prior to introduction of solute.

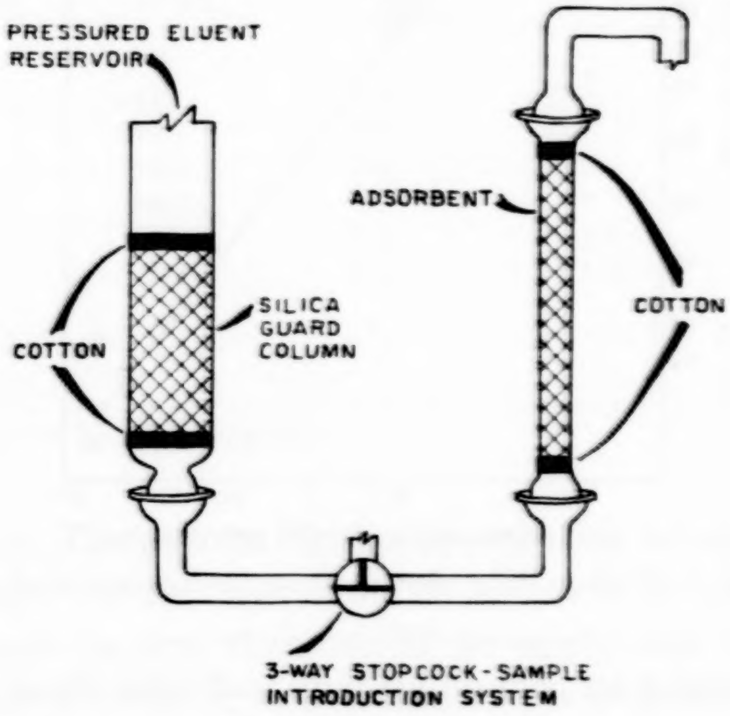

PRESSURED ELUENT RESERVOIR
ADSORBENT
SILICA GUARD COLUMN
COTTON
COTTON
3-WAY STOPCOCK-SAMPLE INTRODUCTION SYSTEM

Fig. 1. Apparatus.

One example will demonstrate both the experimental steps used in determining solute retention volumes and the equivalence of linear retention volumes so obtained to corresponding equilibrium distribution $\underline{K}$ values. A 10 mm × 75 mm glass column was filled with 2.07 g of "nominal" 2.5% H_2O–Al_2O_3. A known weight of a solute, azulene, was introduced into the column at the three-way stopcock, and this was eluted through the column with pentane. Eluent fractions of measured volume were collected. The concentration of azulene in these fractions was determined by ultraviolet spectroscopy in the 270 to 353 mμ region. From these data the cumulative weight percent solute removal from the column was plotted against the cumulative eluent volume. The retention volume R', defined as the eluent volume required to elute 50% of the solute, can be interpolated from the above plot, as shown in Fig. 2; this plot is on arithmetic probability graph paper. The Gaussian elution band predicted by theory should give a linear plot in the latter case; most experimental adsorption elution cases show band asymmetry (and non-linearity of the plot of Fig. 2), however, persisting for column loadings *well below* those required for retention volume linearity. This suggests that the frequently reported practice of interpreting chromatographic linearity from band shape is ill advised. The column void volume V_0 is equal to the retention volume obtained when iso-octane is solute, using refractive index to follow elution. The corrected solute retention volume R was then obtained as $R' - V_0$. Finally, division of this quantity by the adsorbent weight gave the equivalent retention volume $\underline{R}$ for azulene. Using this technique with varying amounts of the solute azulene for the above column, a number of experimental values of $\underline{R}$ can be obtained which correspond to different solute to alumina ratios, W_s. In addition, the equilibrium measurement of solute distribution coefficients $\underline{K}$ for the same solute, eluent, and adsorbent system was also carried out, with the results shown in Table I. For the equilibrium data, an average value of the distribution coefficient $\underline{K}$ of 6.5 ml/g is found, in good agreement with the average of the chromatographic values (6.7 ml/g) in the linear region (for W_s values less than $120 \cdot 10^{-6}$). As previously defined[1], the values of $\underline{K}$ and $\underline{R}$ in the linear concentration region are referred to by superscript zeros, $\underline{K}^\circ$ and $\underline{R}^\circ$; unless otherwise specified, all retention volumes referred to in the present paper are linear equivalent retention volumes, $\underline{R}^\circ$. As a final commentary on the data of Table I, it has previously been shown[1] that the linear extrapolation of non-linear chromatographic data to zero solute concentration

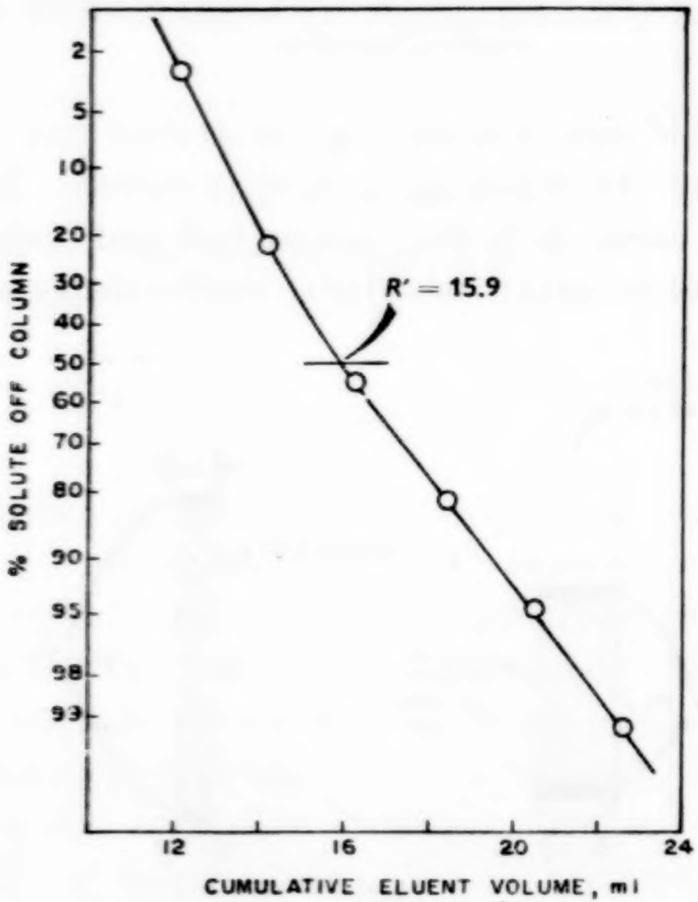


Fig. 2. Elution data plot to show interpolation of R'.

provides correct linear retention volumes *only* under certain conditions. The previous test for these conditions specified that the two non-linear retention volumes extrapolated should differ by no more than 10 % and that the solute column loadings (W_s) for the two retention volume values should differ by at least a factor of 10. This may be rephrased as follows: The percent change in retention volume on going to a lower column loading, divided by the logarithm of the ratio of larger to smaller solute column loadings, should be less than 10. If we apply this test to the non-linear chromatographic values of Table I, we see that the two $\underline{R}$ values for W_s equal $300 \cdot 10^{-6}$ and $120 \cdot 10^{-6}$ do not meet this condition, since the percentage increase in retention volume (9 %) divided by the log of the ratio of W_s values (0.40) is greater than 10 % (22 %). The next pair of retention volumes, for W_s equal $120 \cdot 10^{-6}$ and $30 \cdot 10^{-6}$, essentially meet the condition, the percentage increase in retention volume per logarithm W_s ratio equaling 11 %. In the latter case, the linear extrapolation of $\underline{R}$ to W_s equal zero gives a value of $\underline{R}^\circ$ equal to 6.6, which is quite close to the observed value in the linear region. For the former pair of $\underline{R}$ values, linear extrapolation gives a value of $\underline{R}^\circ$ equal to 6.4, which is significantly lower than the average of linear $\underline{R}$ values. Since neither of these pairs of values fails the extrapolation condition severely, the extrapolated value of $\underline{R}^\circ$ is close to the observed values in each case.

TABLE I

EQUILIBRIUM SOLUTE DISTRIBUTION COEFFICIENTS AND CHROMATOGRAPHIC EQUIVALENT RETENTION VOLUMES AS A FUNCTION OF SOLUTE CONCENTRATION FOR THE SYSTEM AZULENE (SOLUTE)–*n*-PENTANE–2.3 % H_2O–Al_2O_3

Equilibrium data Solute concentrations			*Chromatographic data*	
Non-sorbed phase × 10^6 *g/ml*	*Adsorbed phase* × 10^6 *g/g*	$\underline{K}$ *(ml/g)*	W_s × 10^6 *g/g*	$\underline{R}$ *(ml/g)*
66.4	443	6.7	300	5.6
23.1	139.2	6.0	120	6.1
5.60	35.9	6.4	30	6.5
2.30	14.1	6.1	12	6.8
0.552	3.61	6.5	3.6	6.8
0.212	1.52	7.2	1.2	6.7
Average		6.5		6.7 ± 0.1*

* Average of last four values.

Counter gravity pressured flow was maintained in every case, as implied by the equipment diagram of Fig. 1. All experiments were carried out at room temperature (24 ± 1°).

Alcoa F-20 grade alumina was first calcined at 400° for 8 to 16 h, and adsorbent samples of various "nominal" activities prepared by the addition of known amounts of water to weighed samples of the dry adsorbent. The samples were kept in rubber-stoppered flasks and were permitted to equilibrate for at least 24 h prior to use. This procedure appeared to give samples of reproducible activity, when the alumina was from the same batch. There is some indication, however, that adsorbent "activity", even after calcining, varies slightly with different batches. Because of this, we have defined adsorbent activity in terms of the $\underline{R}^\circ$ value for a standard solute–eluent combination. Fig. 3 shows $\underline{R}^\circ$ values for naphthalene as solute and *n*-pentane as eluent as a function of the percent water added to a calcined sample of alumina. For adsorbent with less than 1.0% added water, linearity occurs for this particular solute only at very low column loadings, (W_s values). These $\underline{R}^\circ$ values were themselves determined at a constant column loading ($W_s = 5 \cdot 10^{-6}$) which represents a compromise between linearity and detection of the eluted solute. All of the data of the present paper report adsorbent activities in terms of percent water contained, this value being determined experimentally by this correlation. The extension of the adsorbent deactivation scale of Fig. 3 into negative values reflects the slight variation of activity in starting calcined adsorbent samples.

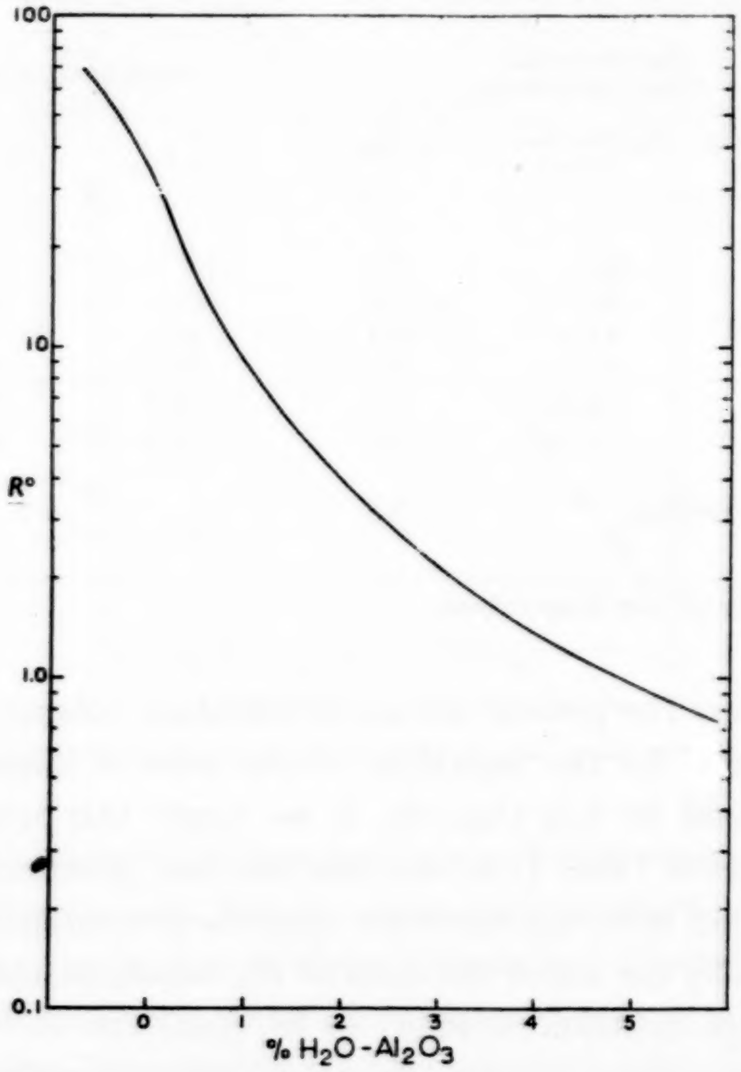


Fig. 3. $\underline{R}^\circ$ for $C_{10}H_8$ as a function of adsorbent activity; *n*-pentane eluent.

Continued elution of a chromatographic column can lead to changes in the average activity of the adsorbent, as measured by a standard $\underline{R}^\circ$ value. With calcined and lightly deactivated aluminas, adsorbent deactivation occurs rapidly unless the eluents used are scrupulously purified prior to use. In the present studies, reagent-grade or 99+% eluents were first purified by passage over silica gel, and the apparatus of Fig. 1 is seen to contain a silica gel guard column for further purification of eluent during use. As a further precaution, when the same column was used for several elution runs, the column activity was frequently checked by means of the measurement of R° for a standard solute. For alumina samples containing more than 4 to 5 % water, use of strong eluents such as benzene leads to a very rapid increase in adsorbent activity (to that of 4–5 % H_2O–Al_2O_3), presumably due to the removal of non-chemically bound water. The same phenomenon occurs less rapidly in the case of saturate eluents. As a result, the use of aluminas containing more than 5 % water is excluded with eluents stronger than pentane, and is limited even for the saturate eluents.

It has been observed in isolated examples that when eluent flow rate is increased much above 150 ml/min, values of R° begin to increase above the constant value observed at lower flow rates. Consequently, all R° values reported in the present and previous papers were obtained for flow rates below this critical value. It is assumed that non-equilibrium conditions associated with the higher rates are responsible for this dependence of retention volume values on the higher flow rates.

The solutes used were either the best commercial samples available, or gifts from various individuals and institutions, and not further purified. Purity is of limited importance in the determination of R° values, providing that the impurities are not present in major ($\sim$ 50 % wt.) amounts and do not have closely similar adsorption and ultraviolet absorption properties.

THE PREDICTION OF LEAC RETENTION VOLUMES

The following sections present the systematic development of a mathematical model for the correlation of solute retention volume data in LEAC systems with experimental conditions and solute structure. The model permits the quantitative prediction of R° values for alumina as adsorbent when the values of certain experimental parameters are known. With certain modifications, the model should be applicable to other adsorbent systems, but this will provide the subject for future communications. Before considering the detailed mathematical development of the model under discussion, the final correlational equation will first be presented, following which some examples of its application will be given. Eqn. (1) relates the logarithm of a linear equivalent retention volume to adsorbent surface volume V_a, proportional to adsorbent surface area, an adsorbent activity function α, eluent polarity or eluting power ε°, solute size $\sum^i \delta_i$, solute structural groups $Q^\circ{}_i$, and solute geometry terms $q^\circ{}_j$.

$$\log R^\circ = \log V_a + \alpha \left[\sum^i Q^\circ{}_i + \sum^j q^\circ{}_j - \varepsilon^\circ \sum^i \delta_i \right] \qquad (1)$$

As will be shown, $\log R^\circ$ is proportional to an energy of adsorption, and is equivalent to the log retention volume of gas chromatography and R_M of paper chromatography. The adsorbent surface volume V_a can be easily measured by the BET technique in the case of calcined adsorbents, but the similar determination of V_a for partially deactivated adsorbents presents experimental difficulties and we have used an alternative procedure in the present paper. Table II presents values of V_a for a typical Alcoa F-20 activated alumina as a function of water deactivation. α is an adsorbent activity parameter, ranging in the case of alumina from values of 1 for calcined material to smaller values for deactivated samples. Values of α as a function of percent water deactivation are shown in Table II, again for a particular Alcoa F-20 alumina. Alumina samples with surface areas comparable to this sample may be treated as equivalent by measuring the activity (% H_2O–Al_2O_3) in terms of the experimental $\underline{R}^\circ$ value for naphthalene eluted by pentane, as discussed in the experimental section. These standard $\underline{R}^\circ$ values for the adsorbent in question as a function of water deactivation are also listed in Table II. Each structural group i in the solute contributes a term Q°_i to $\underline{R}^\circ$, as seen in the summation $\sum^{i} Q^\circ_i$ of eqn. (1). These structural groups are defined exclusive of hydrogen for simplicity. Some experimental values of Q°_i are listed in Table III for various groups i. Where the geometry of the molecule affects $\underline{R}^\circ$, a term q°_j must be introduced. Some examples of this effect, along with experimental values of q°_j are also provided in Table III.

TABLE II

ALUMINA ACTIVITY FUNCTION AND ADSORBED VOLUME AS A FUNCTION OF PERCENT WATER DEACTIVATION

% H_2O-Al_2O_3	Activity function α	Adsorbed volume V_a	Standard solute retention volume*
0.0	1.00	0.038	35
0.5	0.90	0.033	16
1.0	0.84	0.029	9.0
1.5	0.79	0.025	5.8
2.0	0.75	0.022	4.0
3.0	0.69	0.018	2.2
4.0	0.63	0.015	1.4
5.0	0.57	0.013	0.97

* Value of $\underline{R}^\circ$ for naphthalene solute, n-pentane eluent.

The eluent polarity parameter ε° is defined as zero for n-pentane (and probably other saturate eluents as well), and increases for more polar eluents. As seen in Table III, eluent polarity increases in the order pentane, carbon tetrachloride, benzene. The molar volume of the solute $\sum^{i} \delta_i$ is determined by adding up the contributions shown in Table III, from individual solute atoms i. In calculating solute molar volume, only those groups or atoms which are confined to the adsorbed phase can be counted. Thus, for alkyl substituents on an aromatic nucleus, $\sum^{i} \delta_i$ equals one, since only the methylene group attached to the ring is confined to the plane of the aromatic ring and hence to the adsorbed phase.

An exception to eqn. (1) exists in the case of the elution of aromatic hydrocarbons by benzene. As eqn. (1) stands, $\underline{R}^\circ$ for aromatic hydrocarbon solutes would be log V_a in this case, since ε° for benzene (0.32) is essentially the same as Q°_i for aromatic carbon (0.31). Actually, experimental $\underline{R}^\circ$ values are small but measurably larger than log

TABLE III

SOLUTE GROUP SEPARATION FACTORS

Group i[a]	Q°_i
Aliphatic carbon (for saturate eluents only)	0.020
Aromatic carbon	0.31
Aromatic sulfur, as in thiophene ring	0.76
Aliphatic sulfur, as in alkyl sulfide	2.6
Aromatic carbonyl, as in benzaldehyde	3.3
Basic aromatic nitrogen (as in pyridine)	4.8
Non-basic aromatic nitrogen (as in pyrrole, carbazole, etc.)	4.2
Aromatic fluoro (as in fluorobenzene)	0.11
Aromatic chloro	0.20
Aromatic bromo	0.33
Aromatic iodo	0.51
Aliphatic chloro (as in benzyl chloride)	1.11
Aromatic ether, as in anisole	1.7
Aromatic ester, as methyl benzoate	2.4
Second order effects j	q°_j
Methyl substituent on aromatic or thiophene[b]	0.04
Alkyl substitution on thiophene ring	— 0.039
Alkyl substitution on N-containing ring of nitrogen aromatic compound	0.5[d]
Alkyl substitution *ortho* to N of aromatic nitrogen compound	— 0.7[d]
Alkyl substitution on ring adjacent to nitrogen-containing ring	0.3[d]
Alkyl iso branch	
Isolated from aromatic ring	— 0.045
Adjacent to ring	— 0.13
Ortho, ortho di-alkyl substitution in thiophenes[c]	— 0.09
Vicinal alkyl substitution on aromatic ring (for each substituent in excess of the first one)[c]	0.09
Naphthene ring closure	0.08
Halogen substituent in naphthalene	— 0.37
m-Halo substituents and *o,p*-substituents past the second one	ignore
Eluent parameters	ε°
n-Pentane	0.00
Carbon tetrachloride	0.13
Benzene	0.32
Atom	*Values of* δ_i
Hydrogen	0
First-row elements (C, N, O, F, etc.)	1
Second-row elements (P, S, Cl, etc.).	3

[a] We have chosen to define the group *i* both with respect to its constituent atom(s), exclusive of hydrogen, and to its nearest neighbor atoms.

[b] Does not include Q°_i for one aliphatic carbon.

[c] In addition to normal substituent effect.

[d] Assumes ε° for 50% benzene–iso-octane equal 0.20.

V_a. The reason for this discrepancy is discussed in the following text. Eqn. (1) must be modified for this particular case as follows. A value of Q°_i for each aromatic carbon is assumed equal to 0.14, and the actual number of aromatic carbons n in the solute is decreased by six before adding the Q°_i terms; $\sum^i Q^\circ_i$ is therefore 0.14 (n—6). In addition ε° is set equal to zero.

A few examples of the application of eqn. (1) and the data of Tables II and III to the prediction of $\underline{R}^\circ$ values for specific cases will be offered. First, assume it is desired to calculate $\underline{R}^\circ$ for the solute chrysene, using n-pentane elution from 3.9% H_2O–Al_2O_3. There are 18 aromatic carbons in this compound, so that $\sum^i Q^\circ_i$ equals 18 × 0.31 or 5.58. No second order (q_j) terms for the unsubstituted aromatic hydrocarbons are shown in Table III (although a later communication will show their existence and discuss their origin), so that $\sum^j q^\circ_j$ equals zero. For pentane as eluent, ε° is equal to zero, so that the last term of eqn. (1) also vanishes. From Table II, values of a (0.634) and of V_a (0.0153) may be interpolated for 3.9% H_2O–Al_2O_3. Substitution of these various terms into eqn. (1) then gives:

$$\begin{aligned}\log \underline{R}^\circ &= \log (0.0153) + 0.634\,(5.58 + 0 - 0)\\ &= 1.71\end{aligned}$$

In this case, an experimental value was found equal to 1.72.

For the elution of the same solute (chrysene) from the same adsorbent (3.9% H_2O–Al_2O_3) by carbon tetrachloride, only the eluent term $\varepsilon^\circ \sum^i \delta_i$ changes. For this eluent, $\varepsilon^\circ = 0.13$, and since there are 18 carbon atoms in the solute which must lie in the adsorbed phase, $\sum^i \delta_i = 18$. Substituting this new value of $\varepsilon^\circ \sum^i \delta_i$ (2.34) into the above calculation based on eqn. (1) gives:

$$\begin{aligned}\log \underline{R}^\circ &= \log (0.0153) + 0.634\,(5.58 + 0 - 2.34)\\ &= 0.23;\end{aligned}$$

an experimental value equal to 0.38 was found. The elution of 3,4-benzpyrene by benzene from 1.6% H_2O–Al_2O_3 represents an exception to the direct application of eqn. (1). As previously discussed, the structural groups counted are the 20 aromatic carbons of the solute, less the 6 of the eluent, or 14. A value of Q°_i equal to 0.14 is assumed, and the eluent terms are ignored. Substitution of the appropriate terms into eqn. (1) now gives:

$$\begin{aligned}\log \underline{R}^\circ &= \log (0.0244) + 0.782\,(1.96 + 0)\\ &= -\,0.08.\end{aligned}$$

The experimental value found was — 0.10.

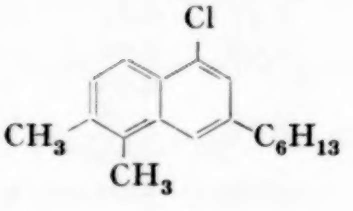
In the elution of 2,6-dimethylpyridine from 4.25% H_2O–Al_2O_3 by benzene, the Q°_i terms include two alkyl carbons (0.04), five aromatic carbons (1.55), and a basic nitrogen (4.8), for a total of 6.39. The q_j terms include two alkyl substituents on a nitrogen-containing ring (1.0), and two *ortho* alkyl substitutions to the N atom (— 1.4), for a total of — 0.4. There are eight first-row atoms in the solute, so $\sum^i \delta_i$

equals 8, and ε° for benzene as eluent equals 0.32. Inserting these and the other appropriate terms (from Table II) into (1) gives:

$$\begin{aligned}\log \underline{R}^\circ &= \log (0.0145) + 0.615 (6.39 - 0.4 - 2.56)\\ &= 0.26.\end{aligned}$$

The experimental value found was 0.34.

Consider finally the elution of the hypothetical solute 1-chloro-3-*n*-hexyl-5,6-dimethyl-naphthalene (I) from 3.2% H_2O–Al_2O_3 by carbon tetrachloride.



(I)

The $Q^\circ{}_i$ terms for the solute include 10 aromatic carbons (3.1), one aromatic chloro atom (0.20), and the three aliphatic carbon atoms (0.06) which are confined to the plane of the solute nucleus (it is assumed that for an eluent more polar than pentane, the five terminal carbons of the *n*-hexyl group will lie in the eluent phase). The first-order substituent terms total 3.36. The second-order $q^\circ{}_j$ terms include the correction for the chloro substitution in naphthalene relative to benzene (— 0.37), two aromatic methyls (0.08), and *two* vicinal alkyl groups (0.18) since the α-methyl group in naphthalene is considered *ortho* to the adjacent 8-hydrogen. The total of the $q^\circ{}_j$ terms is seen equal to + 0.15. There are 13 first-row atoms of the solute confined to the adsorbed phase, and one second-row atom for a total $\sum^i \delta_i$ of 16; $\varepsilon^\circ \sum^i \delta_i$ is therefore 2.08. Inserting the other parameter values of Tables II and III into eqn. (1) we have:

$$\begin{aligned}\log \underline{R}^\circ &= \log (0.017) + 0.68 (3.36 + 0.15 - 2.08)\\ &= -0.79.\end{aligned}$$

The limited group factor ($Q^\circ{}_i$) data of Table III pose an obvious restriction on the range in solute types for which retention volume may presently be predicted. In addition, even for the solute types considered, some simplification in the $q^\circ{}_j$ terms has been effected in Table III. Thus, no notice is taken of the effect of size and structure of the alkyl substituent *ortho* to ring nitrogen or thiophene sulfur atoms. In both cases, the following data of Tables IX and X suggest that as the *ortho* groups get bulkier, the retention volumes will fall below those predicted by the parameters of Table III. These steric effects will no doubt be highly specific, and there may be some question of our ability to make quantitative correlations in this area.

The accuracy of eqn. (1) as a prediction tool is conditional, as indicated above, on the inclusion of all important $q^\circ{}_j$ terms. Beyond this, the relationship may be described as semi-quantitative. Tables IV and V show some examples of its reliability in the case of the aromatic hydrocarbons and two non-basic nitrogen compounds. Subsequent tables present a number of additional experimental $\underline{R}^\circ$ values, expressed as relative retention volumes $\underline{R}^*$ ($\underline{R}^\circ$ for substituted aromatic divided by that of

TABLE IV

EXPERIMENTAL AND CALCULATED $\underline{R}^\circ$ VALUES
FOR AROMATIC HYDROCARBONS OVER A RANGE IN CONDITIONS

Number of aromatic carbons in solute	Log $\underline{R}^\circ$							
	n-Pentane 1.2% H_2O-Al_2O_3		n-Pentane 4.0% H_2O-Al_2O_3		CCl_4 4.0% H_2O-Al_2O_3		Benzene calcined alumina	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
6 (Benzene)	0.09	— 0.05	— 0.58	— 0.65				
10 (Naphthalene)	0.92	0.97	0.15	0.13	— 0.68	— 0.69	— 0.86	— 0.86
12 (Acenaphthylene)	1.43	1.47	0.54	0.52	— 0.51	— 0.46	— 0.52	— 0.58
14 (Phenanthrene,	2.05	1.98	0.91	0.91	— 0.21	— 0.23	— 0.29	— 0.30
anthracene)	2.12		0.95		— 0.20		— 0.10	
16 (Pyrene,	2.37	2.49	1.18	1.30	— 0.03	— 0.01	— 0.07	— 0.02
fluoranthene)	2.57		1.29		0.05	— 0.01	0.01	
18 (Triphenylene,	2.87	3.00	1.64	1.69	0.38	0.22	0.26	0.26
chrysene)	3.05		1.72		0.38		0.40	
20 (Benzpyrene)			2.20	2.08	0.58	0.44	0.57	0.54

TABLE V

EXPERIMENTAL AND CALCULATED LOG $\underline{R}^\circ$ VALUES
FOR INDOLE AND CARBAZOLE OVER A RANGE IN CONDITIONS

Solute	% H_2O-Al_2O_3	Eluent	Log $\underline{R}^\circ$	
			Experimental	Calculated
Indole	1.6	Benzene	1.10	1.46
Indole	3.0	Benzene	0.84	0.95
Indole	5.0	Benzene	0.23	0.33
Indole	4.8	Pentane	2.18	2.01
Carbazole	1.6	Benzene	1.56	1.46
Carbazole	3.0	Benzene	0.98	0.95
Carbazole	4.0	Benzene	0.56	0.64
Carbazole	5.0	Benzene	0.15	0.33
Carbazole	4.8	Pentane	2.54	2.73

unsubstituted aromatic). When the parameter values of Tables II and III are used with eqn. (1) to calculate these data, the average difference or error between experimental and calculated values is as follows.

Substitution class	Average error (log units)	Range in experimental values (log units)	Number of solutes
Halo aromatics (Table VII)	0.04	1.02	18
Alkyl benzenes (Table VIII)	0.02	0.78	35
Alkyl thiophenes (Table IX)	0.02	0.38	25
Alkyl pyridines* (Table X)	0.07	0.83	12

* Excluding 3-methyl-4-ethyl-pyridine.

The extension of the present model to the interpretation of other adsorbent chromatographic systems appears justified in view of its relative success in the correlation of retention volume data for alumina.

Dependence of retention volume on solute structure and adsorbent activity

Consider a general solute molecule, *x-y-z-*, composed of structural elements or groups *x*, *y*, *z*, etc., which is present in small concentration in an eluent–adsorbent system. At equilibrium a competition will exist between the solute and the eluent E for a place in the adsorbed phase. This equilibrium can be written:

$$x\text{-}y\text{-}z\text{-}_{(n)} + rE_{(a)} \rightleftharpoons x\text{-}y\text{-}z\text{-}_{(a)} + rE_{(n)} \,. \tag{2}$$

The subscripts (n) and (a) refer, respectively, to non-sorbed and adsorbed phases. Eqn. (2) implies an adsorbed phase of constant volume (monomolecular layer), with r given as the volume ratio of solute to eluent. This assumption is in agreement with the observation of BACHMAN[9] and others[10]. The equilibrium constant K° for eqn. (2) will be determined by the partial molal free energies of molecules *x-y-z-* and E in the adsorbed and non-sorbed phases. For the transfer process eqn. (2),

$$F_t = (F_{xyz})_a + r\,(F_E)_n - (F_{xyz})_n - r\,(F_E)_a,$$

with F_t referring to the net transfer free energy divided by 2.3 RT and $(F_i)_j$ referring to the partial molal free energy ($\div$ 2.3 RT) of component i in phase j; $K^\circ = 10^{-F_t}$.

As a first approximation $(F_{xyz})_j$ will be expressible as a linear combination of structural group free energy terms, $(F_x)_j$, $(F_y)_j$, etc., so that:

$$(F_{xyz})_j = (F_x)_j + (F_y)_j + (F_z)_j \tag{3}$$

Two obvious limitations on eqn. (3) exist. First, the phase j must be strictly comparable or constant; each structural group i must "see" a constant molecular environment outside the solute molecule. For a given *linear* chromatographic system (same eluent and adsorbent) this limitation is unimportant since adsorbent coverage, θ, is small. Second, the structural group i must similarly "see" the same internal molecular surroundings. When the electronic and/or steric interaction of the remainder of the solute molecule with i is changed, $(F_i)_j$ must change accordingly.

Corresponding to the volume equivalence of r molecules of eluent to one molecule of solute, a similar equivalence of some fractional volume of an eluent molecule to a group i can be hypothesized. This permits the formulation of the group transfer equilibrium:

$$i_{(n)} + \delta_i\, E_{(a)} \rightleftharpoons i_{(a)} + \delta_i\, E_{(n)}\,,$$

with net free energy ($\div$ 2.3 RT) F_{iE} equal to:

$$F_{iE} = (F_i)_a + \delta_i\,(F_E)_n - (F_i)_n - \delta_i\,(F_E)_a \,. \tag{4}$$

For the solute *x-y-z-*, F_t is equal to $\sum^{i} F_{iE}$.

The relationship between the thermodynamic equilibrium constant K° and the distribution coefficient $\underline{K}^\circ$ previously defined is:

$$\underline{K}^\circ = V_a\, K^\circ\,, \tag{5}$$

from which:

$$\underline{R}^\circ = V_a K^\circ \tag{5a}$$

The quantity V_a refers to the adsorbent capacity, or volume of the adsorbed phase per unit weight of adsorbent; it is required to make the concentration units in the adsorbed and non-sorbed phases comparable. If the dimensionless parameter $Q_i = -F_{iE}$ is defined, then the relationship between chromatographic separability and molecular structure is given by:

$$\log \underline{R}^\circ = \log V_a + \sum^{i} Q_i \tag{6}$$

and for the special case where the solute *xyz*- is composed of *n* equivalent groups, *k*,

$$\log \underline{R}^\circ = \log V_a + n\, Q_k \tag{7}$$

In Fig. 4, the applicability of eqn. (7) is illustrated for calcined alumina as adsorbent, unsubstituted aromatic hydrocarbons (or olefins) as solutes and three different eluents. In each case the unsaturated carbon atoms are taken as structural groups *k*.

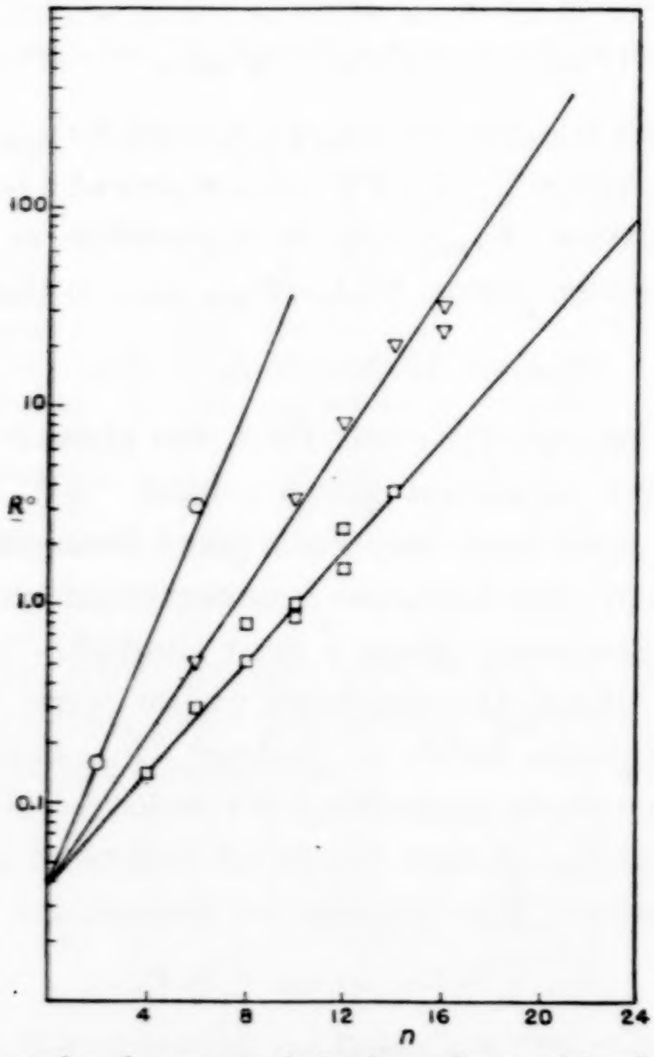


Fig. 4. $\underline{R}^\circ$ *versus* carbon number for aromatic hydrocarbons and calcined alumina. Eluent: ○ *n*-pentane; ▽ carbon tetrachloride; □ benzene.

For pentane and CCl_4 as eluents, the value of *n* for eqn. (7) is set equal to the total number of these unsaturated carbon atoms in the molecule, while for benzene as eluent, the value of *n* taken is the total number of aromatic carbons less 6, since in this case the eluent (for which $\underline{R}^\circ$ must equal V_a) is a member of the solute series with $n = 6$ (this latter adjustment of n will be considered in greater detail in another part of this paper). It is seen that the data for each eluent are linear in n, within a certain scatter, and that the various curves intersect at a common point on the n equal zero axis, as required by eqn. (7). A value of 0.04 ml/g for V_a can be inferred from the data of Fig. 4. This value is in good agreement with the previous value[10] of 0.05 ml/g obtained from the equilibrium uptake of 1,2,3,5-tetramethylbenzene by calcined alumina. Measurement of the same quantity by the BET technique (uptake of nitrogen at — 195°) gives a value of 0.054 ml/g (equivalent to a surface area of 156 m^2/g), also in agreement with the value obtained from Fig. 4. Fig. 5 shows a plot of experimental values of V_a for alumina (obtained by the extrapolation procedure of Fig. 4) *versus* the extent of water deactivation.

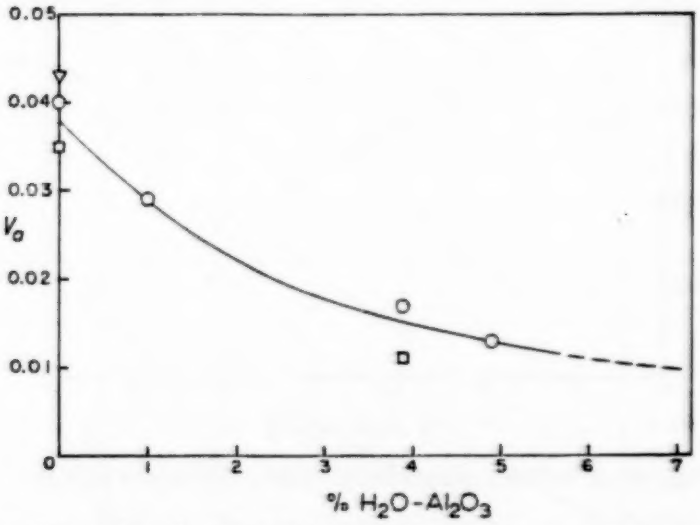


Fig. 5. Variation of V_a with adsorbent activity. Eluent: ○ pentane; ▽ carbon tetrachloride; □ benzene.

Eqn. (7) should also describe the retention volume data for an eluent molecule substituted by n equivalent groups; *e.g.*, for the elution of substituted benzenes by benzene. SPORER AND TRUEBLOOD[5] have reported retention volume data for a number of substituted benzenes, using silicic acid as adsorbent and benzene as eluent. Their calculated values of K_{ph} (the equivalent of our value of 10^{Q_i} for the benzene ring) are therefore equivalent to V_a. These values of K_{ph}, excluding anomalous data for the methoxy and biphenyl derivatives, give an average value of 0.3 ± 0.1. Comparison with the value of 0.05 for alumina shows an adsorbed phase volume V_a substantially larger for silicic acid than for alumina; this is in agreement with surface area measurements for "average" samples of these two adsorbents (*e.g.*, Davison chromatographic-grade silica shows V_a equal to 0.29 ml/g by BET). These authors' assumption that $\underline{R}^\circ$ for the biphenyl solutes should be equal to K_{ph} times $\underline{R}^\circ$ for the corresponding benzene derivative is in error, because of the equality of K_{ph} and V_a for benzene as eluent. The observed small reduction in $\underline{R}^\circ$ for the substituted biphenyls relative to the benzenes is primarily the result of the strain energy arising from forcing the biphenyl ring system into planarity upon adsorption, rather than a Q_i term for the phenyl ring, which is theoretically zero. Because this reduction in $\underline{R}^\circ$ is less than V_a, values of K_{ph} calculated from the biphenyl solutes are uniformly high, as can be seen in Table 4 of their paper[5].

The structural group retention factors Q_i decrease regularly with increasing water deactivation of the adsorbent, and an approximate linear free energy relationship appears to exist between Q_i and a function α of the percent water deactivation:

$$Q_i = \alpha Q^\circ_i \tag{8}$$

Q°_i is equal to Q_i for the calcined adsorbent, and hence α must be set equal to 1 for 0.0% H_2O-Al_2O_3. The adsorbent activity function α is shown in Fig. 6 as a function of percent added water. Within the accuracy of eqn. (8) as a correlational equation, a composite relationship may be set down relating solute separability to structure and adsorbent activity:

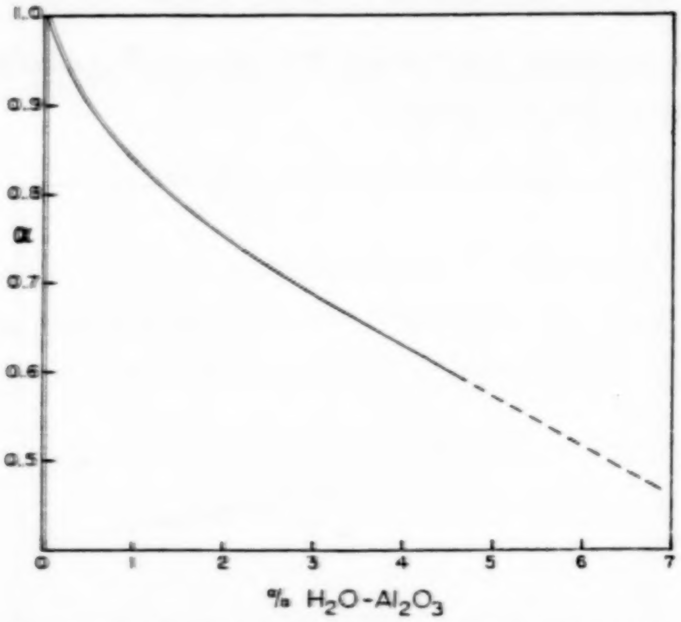


Fig. 6. α *versus* adsorbent water deactivation.

$$\log \underline{R}^\circ = \log V_a + \alpha \sum^{i} Q^\circ_i \tag{9}$$

Fig. 7 shows the family of curves relating retention volume $\underline{R}^\circ$ to adsorbent activity for various values of $\sum^{i} Q^\circ_i$. Within the accuracy of eqns. (6) and (8), retention volume data for a single solute and eluent should fall on one of these curves for alumina as adsorbent. Fig. 7 also shows some typical data in this respect, thus verifying the usefulness of eqn. (9) as a correlational relationship, and the validity of eqn. (8).

To the extent that eqn. (8) is valid, it is seen that for a given eluent, relative solute separability must remain unchanged as adsorbent activity is varied. This might be termed a "constant eluent non-crossing rule". This preservation of separation order is consistent with the removal of the more active sites *without* producing a new phase (water) or a new set of sites of fundamentally different adsorption properties. The regular decrease in the volume of the adsorbed phase (V_a) with increasing adsorbent water content bears this conclusion out, as does the decline with deactivation of α, which is proportional to the average energy (of interaction with solute) of the remaining adsorbent sites. As discussed in the experimental section, when water content exceeds 5%, the excess water is readily eluted by the stronger eluents such as benzene, and more slowly by saturate eluents. This constitutes a further check on the experimental value of V_a (0.04–0.05) for alumina, and also rules out the importance of a water *phase* for adsorbent samples containing less than 5% water.

Previous investigators have emphasized the importance of restricting chromatographic studies such as the present to fully activated adsorbents, in order to ensure systems free of partition effects. The above discussion suggests, at least for alumina as adsorbent, that (a) partition as a phenomenon is unimportant for 0–5% H_2O–Al_2O_3, and (b) measurements made on adsorbent within this compositional range can be quantitatively compared. Experimentally, the study of deactivated adsorbents is frequently preferable to corresponding investigations of fully activated samples for at least two reasons: first, adsorbent *linear* capacity, θ_1, increases markedly with adsorbent deactivation in the case of alumina[1], and probably other adsorbents as well; and second, the measurement of large $\underline{R}°$ values by elution techniques is experimentally inconvenient; use of less active adsorbents can reduce retention volumes to a more desirable range. Of equal importance, as will be seen, the measurement of $\underline{R}°$ values as a function of adsorbent activity (α) offers certain possibilities in the theoretical interpretation of the nature of the adsorbed state.

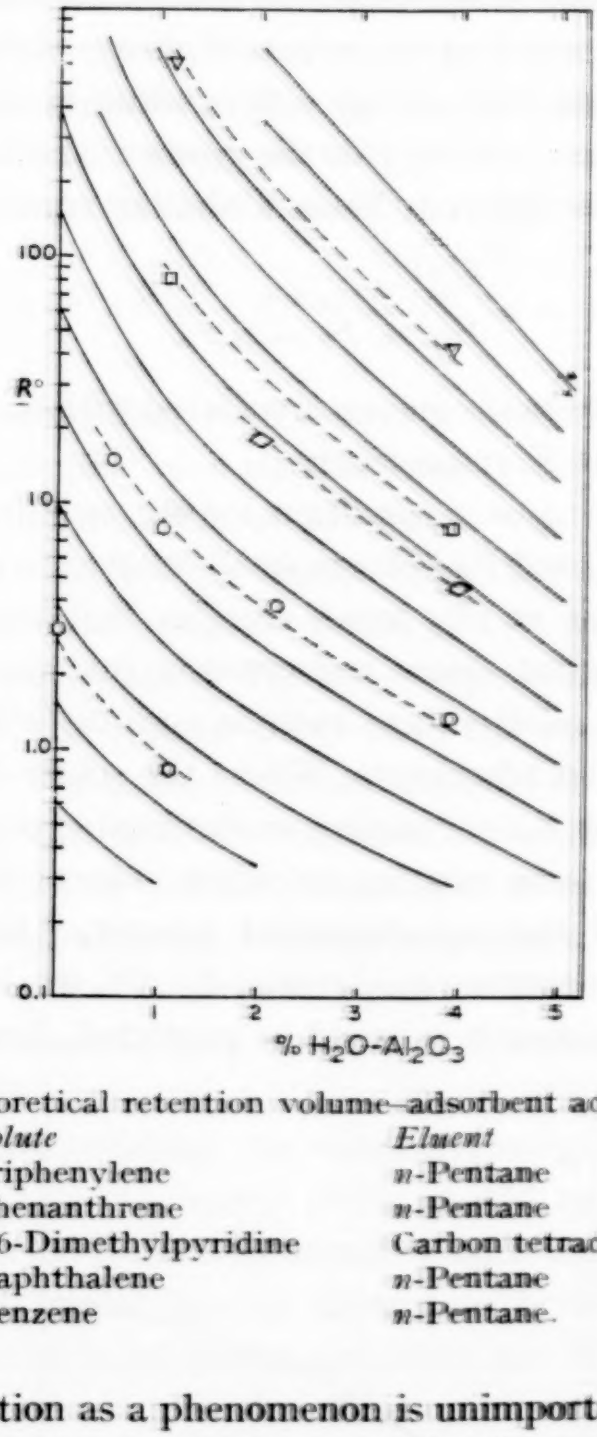


Fig. 7. Theoretical retention volume–adsorbent activity curves.

Solute	*Eluent*
△ Triphenylene	*n*-Pentane
□ Phenanthrene	*n*-Pentane
◇ 2,6-Dimethylpyridine	Carbon tetrachloride
O Naphthalene	*n*-Pentane
⬡ Benzene	*n*-Pentane.

The role of the eluent

The role of the eluent in determining solute retention volumes can be described in terms of eqn. (4), the expression for the group free-energy factors F_{iE}. The solute terms, $(F_i)_j$, can be separated into those energies resulting from solvation forces acting on the group i by eluent, and those resulting from interaction of i with the adsorbent. Since only one side of the molecule is shielded from the non-sorbed phase during adsorption, only a fraction of the solvation energy of the group i will be lost on adsorption. Because the adsorption energy will, in addition, normally be substantially larger than the eluent solvation energy of the group i, the latter energy terms may, to a first approximation, be ignored. From a similar argument for the eluent free energy terms, $-\delta_i\ [(F_E)_a - (F_E)_n]$

$$F_{iE} \approx F_i - \delta_i F_E, \qquad (10)$$

where F_i and F_E refer simply to the energy associated with the formation of adsorbent bonds to group i and eluent E, respectively.

Eqn. (10) can be seen to be a condition for the validity of eqn. (8); when the adsorption energy terms F_i and F_E become small relative to the corresponding eluent solvation terms, differences in the latter energies will begin to determine solute separability, and the constant eluent non-crossing rule would be expected to fail. This further suggests that relationships such as eqn. (8) will be least applicable for the most highly deactivated adsorbents. Where the solute structural group i is the same as those of an eluent i_m, containing m identical groups i, eqn. (10) requires that $Q_i = 0$. Fig. 8 shows some relative retention volume data $\underline{R}^*$ (equal to $\underline{R}^\circ$ for solute divided by that of the unsubstituted aromatic) for the n-alkyl-benzenes C_6H_5-$(CH_2)_{n-1}CH_3$, with n-pentane as eluent and 0.5 % wt. H_2O–Al_2O_3 as adsorbent. No change in $\underline{R}^*$ with variation in n would be predicted. Actually, a modest increase of $\underline{R}^*$ with n is noted (average value of Q°_i for methylene group ≈ 0.020, compared to 0.311 for aromatic carbon), as seen in Fig. 8. Further improvement of the present model would be anticipated by recognition and application of two second-order effects. First, as solute size increases, thus requiring that a larger number of eluent

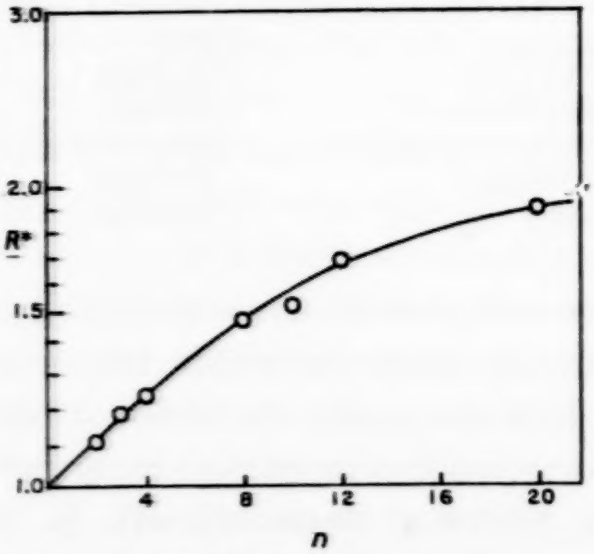


Fig. 8. $\underline{R}^*$ *versus* alkyl carbon number for n-alkyl-benzenes; n-pentane, 0.5 % H_2O–Al_2O_3.

molecules be displaced into the non-sorbed phase, $\underline{R}^\circ$ should increase, all else equal, because of the increase of translational entropy attendant on the substitution of one solute molecule for a larger number of eluent molecules in the adsorbed phase. Second, since the methyl groups of the n-pentane eluent are not exactly equivalent to the methylene groups of the solute side chain, there may be a small difference in adsorption energy. If the latter effect predominates (differing adsorption energies between solute side chain methylene groups and eluent methyl plus methylene groups), then the retention volume ratio for the two solutes of differing side chain lengths should vary with adsorbent activity in the same manner as the group i retention factors. From eqn. (8) and noting the relationship between $\underline{R}^\circ$ and Q_i of eqn. (9) we obtain:

$$\log (\underline{R}^\circ{}_1/\underline{R}^\circ{}_2) = \alpha \log (\underline{R}^\circ{}_1/\underline{R}^\circ{}_2)_{\alpha = 1}$$

If the entropy effect is controlling, then this ratio should be independent of adsorbent activity. For a particular pair of n-alkyl solutes (2-ethyl and 2-n-decyl-thiophene) with n-pentane as eluent, the ratio of $\underline{R}^\circ$ values was found to be 1.59 for 0.5% H_2O–Al_2O_3, and 1.18 for 4.0% H_2O–Al_2O_3. Using the experimental value at 0.5% as reference, the calculated ratio at 4.0% would be 1.59 for the size entropy effect and 1.39 for the adsorption energy effect. The latter appears to predominate, although the calculated magnitude of the change differs from the experimentally observed ratio.

In the correlation of Fig. 4, the structural groups i for the aromatic hydrocarbons were defined as aromatic carbons, with the attendant hydrogen atoms being ignored. The application of eqn. (10) to this case would lead us to predict that Q_i equals zero for the eluent benzene and aromatic carbons as groups i, an observation contrary to fact since the aromatics with carbon number greater than benzene have $\underline{R}^\circ$ larger than V_a. Use of the theoretical model here without qualification probably fails because of the omission of the second-order role of the aromatic hydrogen atoms in determining adsorption energy. Strictly speaking, the structural group of a hydrocarbon should be represented by (CH_x); *i.e.*, the hydrocarbon should be representable as $(CH_x)_n$, not merely by the number of carbon atoms. Under these conditions eqn. (7) will hold for the group (CH_x) regardless of differences between the adsorption energy per volume of aromatic carbons atoms and that for "aromatic" hydrogen atoms. Expressing the higher aromatics as derivatives of benzene, it is seen that each of the benz-derivatives ($C_{10}H_8$, $C_{14}H_{10}$, $C_{18}H_{12}$, etc.) can be rewritten as C_6H_6-$(CH_{0.5})_n$, and this is approximately true of the other higher aromatics as well (*e.g.*, pyrene is C_6H_6-$(CH_{0.4})_{10}$). Now if the adsorption energy per volume of aromatic carbon and hydrogen atoms are different, the displacement of the (hypothetical average) $CH_{0.5}$ group from the adsorbent by some fraction of a CH (benzene) group will be accompanied by a net energy change and Q_i for the $CH_{0.5}$ group will be non-zero. The observation that this value is positive ($Q^\circ{}_i = 0.14$) requires only that the carbon atom have higher adsorption energy per unit volume than the hydrogen atom, a not too surprising discovery. Finally, the retention volumes of the higher aromatics for benzene elution will be determined by the net number of $CH_{0.5}$ groups, which is seen equal to the total number of aromatic carbons less the 6 which make up the benzene "nucleus" of the above formula; thus the correlation of experimental $\underline{R}°$ values in Fig. 4 is not unanticipated. The fact that benzene as a solute also falls on the pentane and carbon tetrachloride curves of Fig. 4 probably arises from the fact that the polarity of aromatic hydrogens is intermediate between the eluents *n*-pentane and carbon tetrachloride and hence Q_i for hydrogen is approximately zero with these two eluents. The latter possibility is theoretically reasonable. The need to subtract 6 from the values of *n* for the benzene eluent data is apparent, since $\underline{R}°$ for the eluent is theoretically equal to V_a.

Returning to eqn. (10), the first order effect on values of $\underline{R}°$ produced by change in eluent may be obtained by taking the difference between values of F_{iE} for the two eluents 1 and 2:

$$\begin{aligned}(F_{iE})_2 - (F_{iE})_1 &= \delta_i\,[(F_E)_1 - (F_E)_2] \\ &= \delta_i\,\varepsilon_{12}\end{aligned} \tag{11}$$

The quantity ε_{12} is proportional to the difference in adsorption energies per volume of the eluents 1 and 2; δ_i is determined by the volume of the group *i*. To a first approximation, the volumes of first-row elements (inferred from their covalent radii[11]) are constant, and those of second-row elements are about three times those of the first row of the periodic table. In addition, the volume of hydrogen is small compared to that of subsequent elements. Relative values of δ_i may thus be set forth as follows:

Atom	*δi (tentative)*
H	0
First-row element (C, N, O, etc.)	1
Second-row element (Si, P, S, etc.)	3

The relative retention volume of a solute in the same adsorbent system using eluents 1 and 2 is given as:

$$\log\,(\underline{R}°_1/\underline{R}°_2) = \varepsilon_{12} \sum^{i} \delta_i \tag{12}$$

Table VI contains retention volume data for a number of solutes, using pentane and benzene as eluents; eluent 1 refers to pentane. Adsorbent activity varied between 3.9 and 4.8% H_2O–Al_2O_3, and retention volume ratios for the two eluents ($\underline{R}°_1/\underline{R}°_2$) have been calculated for a single adsorbent activity (3.9%) by extrapolation of $\underline{R}°$ values by means of the master curves of Fig. 7. In Fig. 9, the logarithm of the ratio of these retention volumes is plotted *versus* $\sum^{i} \delta_i$. This plot clearly shows the dependence of the eluent-induced shift in retention volume on solute size or $\sum^{i} \delta_i$. The linear relationship predicted by eqn. (12) is also approximately confirmed. A value ε_{12} for this eluent pair is found from the slope of Fig. 9, equal to 0.20. Similar data for 3.9% H_2O–Al_2O_3 and the eluent pair pentane (1)–carbon tetrachloride (2) are plotted in Fig. 10, showing a value of ε_{12} equal to 0.08; carbon tetrachloride is thus intermediate between pentane and benzene as regards its adsorption energy per unit volume adsorbed, and eluting "power". In view of the range in molecular structure represented in the solutes of Fig. 9 and Fig. 10, and the anticipated corresponding differences in relative solubility or solvation of these solutes in the two eluents, the

TABLE VI

RETENTION VOLUME DATA FOR VARIOUS SOLUTES USING BOTH *n*-PENTANE AND BENZENE AS ELUENTS

Solute	$\underline{R}^\circ$		$\sum^{i} n_i$	$\underline{R}^\circ_1/\underline{R}^\circ_2$[a]
	n-Pentane	*Benzene*		
Benzpyrene	159[a]	0.24[a]	20	660
Methyl ethyl sulfide	1.02[a]	0.10[a]	5[b]	10
Anisole	3.6[a]	0.12[a]	8	30
Benzaldehyde	30[a]	0.85[a]	8[a]	35
Benzene	0.26[a]	0.015[c]	6	17
2,6-Dimethylpyridine	46[d]	2.2[d]	8	23
Indole	153[e]	1.7[e]	9	120
Carbazole	346[e]	1.4[e]	13	393

[a] 3.9% activity.
[b] Assumes two aliphatic carbons in adsorbed phase (see latter part of present section).
[c] Value of V_a.
[d] 4.2% activity.
[e] 4.8% activity.

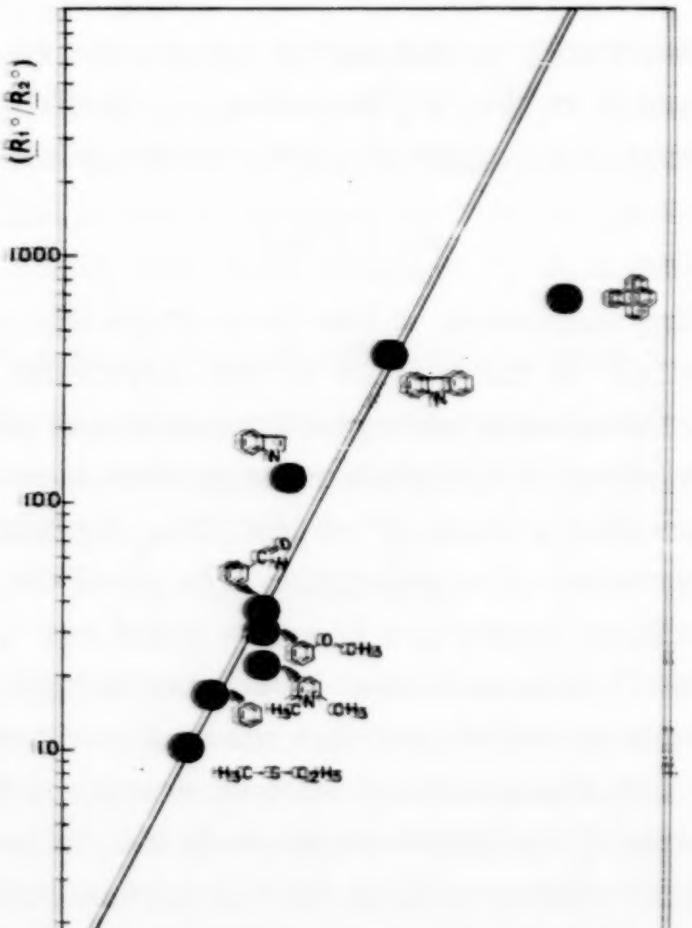


Fig. 9. Dependence of retention volume on eluent as a function of solute size; 3.9% $H_2O-Al_2O_3$, *n*-pentane–benzene eluents.

linear nature of the latter plot tends to confirm the *relative* unimportance of solubility effects in the determination of solute retention volume (as previously predicated). This conclusion is strengthened by the fact that the adsorbent activity in this case is quite low, and solubility effects should be most important at the lower adsorbent activities.

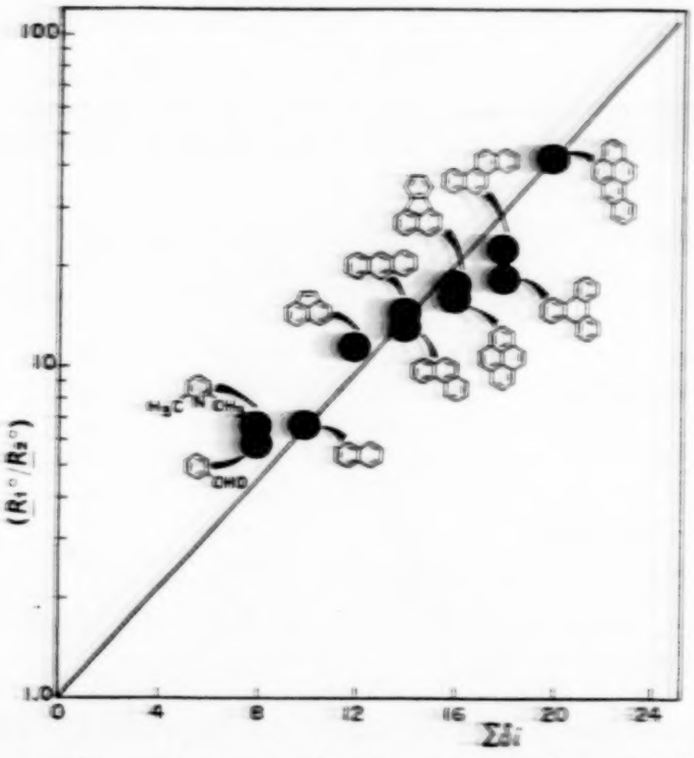


Fig. 10. Dependence of retention volume on eluent as a function of solute size; 3.9% H_2O–Al_2O_3, *n*-pentane–carbon tetrachloride eluents.

Since the adsorption energy of the eluent is expected to change with adsorbent activity in the same manner as that of the solute, ε_{12} should vary with α as does Q_i. Thus, ε_{12} should be related to its value $\varepsilon°_{12}$ for calcined adsorbent as:

$$\varepsilon_{12} = \varepsilon°_{12}\,\alpha \tag{13}$$

Throughout the preceding discussion, it has been implicitly assumed that the total solute molecule is confined to the surface of the adsorbent. For planar aromatics this is necessarily so, *if* the adsorption region approximates planarity. That the total aromatic molecule *is* involved in the adsorption process may be concluded from the data of Fig. 4. These data show a linear $R°$–n plot, thus implying that every additional aromatic carbon atom participates in adsorption. The possibility that only a few of the many aromatic carbons form adsorption bonds is ruled out by the observation that molecules with "reactive" carbons (those possessing a high free valence, and low localization energies) such as anthracene are much less strongly adsorbed than are molecules with more, but less reactive, carbon atoms such as triphenylene (see Table IV). When a portion of the solute molecule is free to lie either in or out of the adsorbed phase, the relative value of $\Sigma\, Q_i$ for the part in question should determine its location. Thus, for the *n*-alkyl benzenes and thiophenes, a positive value of Q_i for the methylene group is observed, and this suggests that a considerable portion of such alkyl groups will be confined to the adsorbed phase. That less than all of the very long alkyl groups will remain in the adsorbed phase is indicated by two observations. First, the plot of experimental log R° values *versus* n for the alkylbenzenes (Fig. 8) declines from the initial linear plot at large values of n, whereas a constant value of Q_i for the methylene group *and* the restriction of the total alkyl group to the adsorbed phase would require linearity over the entire range of this plot (log R° = constant + nQ_i). Secondly, it has been noted that the ratio of R° values for 2-*n*-decylthiophene relative to 2-ethyl-thiophene declines from 1.58 at 0.5% H_2O–Al_2O_3 to 1.18 at 4.5% H_2O–Al_2O_3, whereas the extrapolated value from the former to the latter value by means of eqn. (8) is 1.38. The extrapolation assumes that for each solute the same number of methylene groups will occupy the adsorbed phase for both adsorbent activities. The experimental value of the retention volume ratio at the lower adsorbent activity implies that for decylthiophene only about one-half of the number of methylene groups held in the adsorbed phase at the higher adsorbent activity are so held in the case of the less active adsorbent. This is consistent with the expectation that as Q_i decreases (with decreasing adsorbent activity), for the methylene group, an increasing portion of the terminal carbons in an alkyl chain (particularly for long chains) will be statistically favored to occupy the non-sorbed phase.

In the case of stronger eluents than pentane, Q_i for the methylene group should be negative, the alkyl group of aromatics should lie (except for the ring-attached carbon) in the non-sorbed phase, and the retention volumes of a series of alkyl aromatics should be constant. This does not mean that substituted aromatics should have the same R° values as unsubstituted ones, since the effect of substitution *per se* on the aromatic may be considerable. Experimental data for the 4-*n*-alkyl-pyridines, using 50% benzene–isooctane as eluent and 5.0% H_2O–Al_2O_3, tentatively bear this out. The R° values of the methyl, ethyl, and amyl derivatives stand in the ratio 1.0 : 1.1 : 1.0. Although this is not a sensitive test of the previous assertion, because of the low adsorbent activity and small range in n, it is suggestive of its validity.

Additivity in the adsorption free energy of substituted aromatics

For a substituted aromatic, if the relative retention volume $\underline{R}^*$ is as previously defined (R° for the substituted compound divided by R° for the unsubstituted compound), eqn. (6) requires:

$$\log \underline{R}^* = \sum^{i} Q_i \tag{14}$$

where the summation is over all *substituents i*. SPORER AND TRUEBLOOD[5] have analyzed a number of retention volume data for substituted benzenes eluted from silicic acid by benzene in terms of an expression equivalent to eqn. (14). Table VII presents some relative retention volume data for the halogen-substituted benzenes and naphthalenes, using *n*-pentane as eluent and 0.5% H_2O–Al_2O_3 as adsorbent. It is seen first that Q_i for the halogens as substituents increases with atomic number, or parallels the polarizability of the atom. Deriving values of Q_i from the mono-substituted benzenes (see Table III), it is possible to calculate relative retention volumes for the poly-substituted aromatics. For the *ortho* and *para* substituted di-halobenzenes it is seen that reasonably good agreement between calculated and experimental values exists; the average deviation is only ± 12%. For the one *meta* substituted compound (*m*-dichlorobenzene), the calculated value is substantially too high, and the experimental value is actually less than for the monosubstituted compound. The deviation between calculated and experimental values increases still more in the case of the *ortho-para* substituted tri-halobenzenes, and finally reaches a maximum in the case of the one all *meta tri-halobenzene*, 1,3,5-tribromobenzene. For the 1-halo-naphthalenes, the calculated values are uniformly high by 120 ± 4%. Two second-order effects which affect the validity of eqn. (14) must be recognized. First, resonance interaction of the various substituent groups can result in values of Q_i which vary with substitutional position. Second, only certain substituted isomers (*o,m,p*) may overlap the adsorption sites in an optimum manner. Both of these phenomena have been discussed by SPORER AND TRUEBLOOD[5]. It is difficult to separate the relative importance of these possibilities. Group–ring resonance interaction must be significant, since 1,3,5-tribromobenzene is less strongly adsorbed than is bromobenzene. This implies some deactivation of the nucleus by the second and third bromine groups, without sufficient interaction of these groups with the adsorbent to overcome the effect. Similarly, the 1-halo-naphthalenes are less strongly adsorbed than predicted by a constant adsorption energy increment ($q_j = -0.35 \pm 0.01$). This suggests that the greater number of aromatic carbons in the naphthalene nucleus permit a greater

TABLE VII

RELATIVE RETENTION VOLUME DATA FOR SUBSTITUTED AROMATIC SOLUTES: *n*-PENTANE ELUENT, 0.5% H_2O–Al_2O_3 ADSORBENT

Solute	R^0		*Deviation, %*
	Experimental	*Calculated*	
Benzene	(1.00)	(1.00)	—
Fluorobenzene	1.25	(1.25)	—
Chlorobenzene	1.52	(1.52)	—
Bromobenzene	1.97	(1.97)	—
Iodobenzene	2.88	(2.88)	—
1,2-Dichlorobenzene	2.13	2.31	7
1,4-Dichlorobenzene	2.18	2.31	6
1,2-Dibromobenzene	3.75	3.88	4
1,4-Dibromobenzene	2.80	3.88	39
1,4-Diiodobenzene	10.4	8.3	—20
1,2-Iodobromobenzene	5.9	5.7	—3
1,4-Chlorobromobenzene	2.73	2.99	9
1,3-Dichlorobenzene	1.48	2.31	55
1,2,4-Trichlorobenzene	1.70	3.51	106
1,2-Dichloro-4-bromobenzene	2.24	4.55	103
1,3,5-Tribromobenzene	1.50	7.6	407
2-Chloroethylbenzene	10.3		
Naphthalene	(1.00)	(1.00)	—
1-Chloronaphthalene	0.69	1.52	120
1-Bromonaphthalene	0.92	1.97	114
1-Iodonaphthalene	1.27	2.88	127

deactivation of the ring by a halo substituent, and a corresponding reduction in Q_i for the ring. That the lowering of the naphthalene ring adsorption energy is constant for each substituent is reasonable from the similar values for the Hammett σ function[12] for these three halogen substituents. Conversely, the differing atomic radii of chlorine, bromine, and iodine (0.99–1.33) would be expected to result in differences in the deviation of calculated and experimental $\underline{R}^\circ$ values for the 1-halo-naphthalenes if optimum site overlap were the important consideration. Sporer and Trueblood[5] have interpreted differences in R° values for *meta* and *para* disubstituted benzenes in terms of the optimum coverage of adsorbent sites, and some justification for the importance of this effect can be inferred from the data of Table III. Thus, the all *meta* derivatives in each case have a value of $\underline{R}^\circ$ close to that of the corresponding monosubstituted benzene, suggesting perhaps that only one *meta* substituent can overlap an adsorbent site while the ring is adsorbed. Similarly, all *ortho-para* trisubstituted benzenes have retention volumes approximating those of the corresponding disubstituted benzene, again suggesting that a maximum number of substituent groups can be brought into an optimum overlapping position with respect to the adsorbent sites (in this example, two).

In the case of chloroethylbenzene, Q_i for the chloro substituent (1.01) is much higher than in the case of chlorobenzene (0.20). The same effect has been noted by Sporer and Trueblood[5] in the adsorption on silica of amino and hydroxyl groups, where the adsorption affinity of the aliphatic substituent is much higher than that of the aromatic substituent. Converting their K_i values for these groups to the present Q_i factors ($K_i = 10^{Q_i}$), we have (for SiO_2 as adsorbent)

	Q_i *(aliphatic)*	Q_i *(aromatic)*
$-NH_2$	3.8	1.9
-OH	2.4	1.4

It is seen that these aliphatic substituents contribute about twice as much as the aromatic substituent to the adsorption energy of a solute. Still another example of this general phenomenon can be seen in the comparison of Q_i values for the sulfur atom in dialkyl sulfides and thiophenes. For alumina as adsorbent, and *n*-pentane as eluent, Q°_i is 2.7 in the sulfides and 0.76 in the thiophenes. This effect is attributed to differing resonance interaction of the group in question with the remainder of the solute molecule, similar to the progressive weakening by resonance of the basicity of the nitrogen in the series ammonia, pyridine, pyrrole. While this mechanism for the explanation of these differences in aromatic and aliphatic Q_i values must be important, it is worth noting that site overlap may also contribute, since the aliphatic substituents have greater freedom to adapt to the surface of the adsorbent after the ring is adsorbed.

Steric and other second-order effects in the retention volumes of alkyl-substituted aromatics

Tables VIII, IX, and X present a number of relative retention volume data for alkyl-substituted benzenes, naphthalenes, thiophenes, pyridines and quinolines. A number of second order corrections or interaction effects (similar to those for the *meta* halo-benzenes, tri-halo-benzenes, etc.) can be seen in these examples. Table XI summarizes some of the second-order q_j factors applying to special arrangements of alkyl and aromatic carbon atoms, for specified eluent–adsorbent systems. These q_j values must be added to $\sum^{i} Q_i$ for each solute in the calculation of eqn. (6) and following equations. For the relative retention volume data of Tables IV–VI, only the alkyl Q_i terms must

TABLE VIII

RELATIVE RETENTION VOLUMES OF ALKYL-SUBSTITUTED BENZENES AND NAPHTHALENES FOR 0.5% H_2O–Al_2O_3; *n*-PENTANE ELUENT

Solute	R°		Deviation, %
	Experimental	Calculated	
Benzene	(1.00)	(1.00)	—
Toluene	1.20	1.14	—5
Ethylbenzene	1.11	1.11	0
o-Xylene	1.75	1.57	—10
m-Xylene	1.32	1.30	—2
p-Xylene	1.34	1.30	—4
n-Propylbenzene	1.18	1.17	—1
Isopropylbenzene	0.96	0.88	—8
o-Methyl-ethyl-benzene	1.66	1.53	—8
m-Methyl-ethyl-benzene	1.27	1.26	—1
p-Methyl-ethyl-benzene	1.16	1.26	9
1,2,3-Trimethylbenzene	2.52	2.17	—16
1,2,4-Trimethylbenzene	1.84	1.79	—3
1,3,5-Trimethylbenzene	1.41	1.48	5
n-Butylbenzene	1.24	1.24	0
Isobutylbenzene	1.13	1.24	10
sec.-Butylbenzene	0.93	0.92	—1
tert.-Butylbenzene	1.01	0.92	—9
o-Diethylbenzene	1.47	1.49	2
m-Diethylbenzene	1.20	1.23	3
p-Diethylbenzene	1.15	1.23	7
1,3-Dimethyl-5-ethyl-benzene	1.23	1.44	17
m-Methyl-isopropyl-benzene	1.01	1.01	0
p-Methyl-isopropyl-benzene	0.91	1.01	11
1,2,3,5-Tetramethylbenzene	2.47	2.48	0
1,2,4,5-Tetramethylbenzene	2.49	2.48	0
Pentamethylbenzene	4.25	4.14	—3
Hexamethylbenzene	6.0	5.7	—5
n-Octylbenzene	1.46	1.47	1
n-Decylbenzene	1.52	1.57	3
n-Dodecylbenzene	1.69	1.65	—3
n-Eicosylbenzene	1.90	1.90	0
Indane	1.76	1.77	1
Tetralin	2.10	1.95	—7
Cyclohexylbenzene	1.67	1.60	—4
Benzylbenzene	1.52	1.67	10
Naphthalene	(1.00)	(1.00)	—
1-Methylnaphthalene	1.41	1.38	—2
2-Methylnaphthalene	1.22	1.14	—5
1-*n*-Hexylnaphthalene	1.82	1.65	—9
1-*n*-Butylnaphthalene	1.69	1.50	—11
Acenaphthene	2.18	1.93	—11

be taken into account, since the aromatic terms cancel out for the retention volumes of substituted aromatics relative to the unsubstituted ones. $\sum^{i} Q_i$ for the alkyl terms is calculated from the data of Fig. 8 for the solutes of Tables VIII and IX, and assumed zero for the data of Table X (see later discussion).

The relative retention volumes $\underline{R}^*$ of the alkyl benzenes are the most easily interpreted. It is found that branching adjacent to the ring (as in isopropylbenzene) causes a reduction in $\underline{R}^\circ$ ($q_j = -0.122$), and that shifting of the branching by one or more carbon atoms from the ring (as in isobutylbenzene) reduces this contribution by about two thirds ($q_j = -0.041$). Methyl substitution directly on the ring has a larger effect ($Q_i = 0.057$) than would be expected from the extrapolation of the contribution of larger alkyl groups (in Fig. 9) to n equal 1 ($Q_i = 0.023$). These effects suggest that the α hydrogens are increasing the basicity and adsorption energy of the nucleus, and that alkyl substitution *per se* is reducing nucleus-adsorption energy, probably by forcing the adsorbed solute slightly out of the plane of the adsorbent surface.

It is further observed that *o*-alkyl aromatics invariably have larger $\underline{R}^\circ$ values than the corresponding non-vicinal isomers. An attractive interpretation of this effect (which recalls the higher boiling points of *o*-isomers) is offered by considering the

TABLE IX

RELATIVE RETENTION VOLUMES OF ALKYL-SUBSTITUTED THIOPHENES FOR 0.5 % H_2O–Al_2O_3; *n*-PENTANE ELUENT

Solute	$\underline{R}^*$		*Deviation, %*
	Experimental	*Calculated*	
Thiophene	(1.00)	(1.00)	—
3-Methylthiophene	1.03	1.05	2
2-Ethylthiophene	1.01	1.02	1
2-*n*-Propylthiophene	1.03	1.08	5
2-*n*-Butylthiophene	1.12	1.14	2
2-*n*-Hexylthiophene	1.29	1.26	—3
2-*n*-Heptylthiophene	1.28	1.31	3
2-*n*-Octylthiophene	1.37	1.35	—2
2-*n*-Decylthiophene	1.60	1.44	—10
2-Isobutylthiophene	0.96	1.03	7
2-Isopentylthiophene	1.12	1.09	—3
2-(2-Ethyl-1-butyl)-thiophene	1.11	1.15	4
2,5-Di-isopentyl-thiophene	0.95	1.19	20
2,5-Ethyl-isopentyl-thiophene	0.91	1.10	18
2,5-*n*-Propyl-isopentyl-thiophene	0.90	1.16	22
2,5-Diethylthiophene	0.83	1.04	20
2,5-Ethyl-*n*-propyl-thiophene	0.82	1.10	25
2,5-Ethyl-*n*-butyl-thiophene	0.92	1.16	21
2,5-Ethyl-*n*-hexyl-thiophene	1.12	1.28	13
2,5-Ethyl-*n*-octyl-thiophene	1.25	1.38	10
2,5-Di-*n*-propyl-thiophene	0.87	1.15	24
2,5-*n*-Propyl-*n*-pentyl-thiophene	1.02	1.29	21
2,5-Di-*n*-butyl-thiophene	0.93	1.29	28
2,5-Ethyl-isobutyl-thiophene	0.76	1.06	28
2,5-*n*-Butyl-isobutyl-thiophene	0.83	1.18	30
2,5-Di-isobutyl-thiophene	0.66	1.07	38

rotation around the aromatic carbon–aliphatic carbon bond. Because of steric hindrance, this rotation will be restricted in the gas phase for vicinal substituents relative to non-vicinal substituents. As a consequence, this rotational heat capacity contribution to the total molecular free energy will be such as to increase the free energy of non-*ortho* isomers. It is reasonable to expect that much of this rotational free energy contribution will be lost upon adsorption, and as a result the free energy of adsorption of *ortho* relative to non-*ortho* isomers will be decreased, and $\underline{R}^\circ$ increased.

TABLE X

RETENTION VOLUMES OF THE ALKYL-SUBSTITUTED PYRIDINES AND QUINOLINES FOR 5.0% H_2O–Al_2O_3; 50% BENZENE–ISO-OCTANE ELUENT

Compound	$\underline{R}^\circ$		*Deviation, %*
	Experimental	*Calculated*	
Pyridine	14.6	(14.6)	—
3-Methylpyridine	21.2	23.7	12
4-Methylpyridine	23	23.7	3
4-Ethylpyridine	26	23.7	—9
3,4-Dimethylpyridine	41	46	12
3-Methyl-4-ethyl-pyridine	6.2	46	641
4-Amylpyridine	23	23.7	3
Quinoline	11.5	(11.5)	—
4-Methylquinoline	18.8	18.6	—1
6-Methylquinoline	13.7	13.2	—4
7-Methylquinoline	12.4	13.2	6
2-Methylpyridine	9.6	23.7	147
2,3-Dimethylpyridine	13.3	46	245
2,4-Dimethylpyridine	7.5	38	406
2,5-Dimethylpyridine	10.6	38	258
2-Methylquinoline	8.1	18.6	130
2,6-Dimethylquinoline	9.3	31	233
2-Ethylpyridine	6.1	23.7	289
8-Methylquinoline	2.4	13.2	450
2,6-Dimethylpyridine	6.3	38	503
2,4,6-Trimethylpyridine	9.5	62	552

TABLE XI

SECOND ORDER FACTORS q_i

Group	*Aromatic hydrocarbons**	*Thiophenes**	*Pyridines, quinolines***
Substitution on ring, Ar-R,	0.00	—0.036	0.21 (N ring)
Methyl on ring, Ar-CH_3	0.037	0.037	0.21 (N ring) 0.06 (other)
Iso branch on ring, Ar-CHR_2	—0.122		
o-Alkyl substitution on ring	0.083	(0.083)	
Naphthene ring closure	0.072	(0.073)	
Iso branch not on ring, Ar-R-CH-R_2	—0.041	—0.041	

* 0.5% H_2O–Al_2O_3; *n*-pentane eluent.

** 5.0% H_2O–Al_2O_3; 50% benzene–iso-octane eluent.

In Fig. 11, the relative retention volumes $\underline{R}^*$ are logarithmically plotted *versus* the number of methyl groups for the series of vicinal compounds beginning with toluene and ending with hexamethylbenzene. A linear relationship is observed, permitting a value of q_j per *ortho* attachment of 0.087 to be calculated (after subtraction of the contribution per ring-attached methyl: 0.057). This constant contribution per *ortho* attachment (counted as $n - 1$ for each cluster of n vicinal substituents) suggests that vicinal alkyl groups through intermeshing of hydrogens may possess (in the gas phase) only the rotational heat capacity of one non-vicinal group. The higher $\underline{R}^\circ$ value of 1- as opposed to 2-methyl-naphthalene suggests that substituents in the 1-position of naphthalene (and presumably 1-position of other polyaromatics) behave as *ortho* substituents; the ratio of $\underline{R}^\circ$ values for the methylnaphthalenes is observed to be 1.16, while that calculated on the basis of an *ortho* linkage would be 1.21.

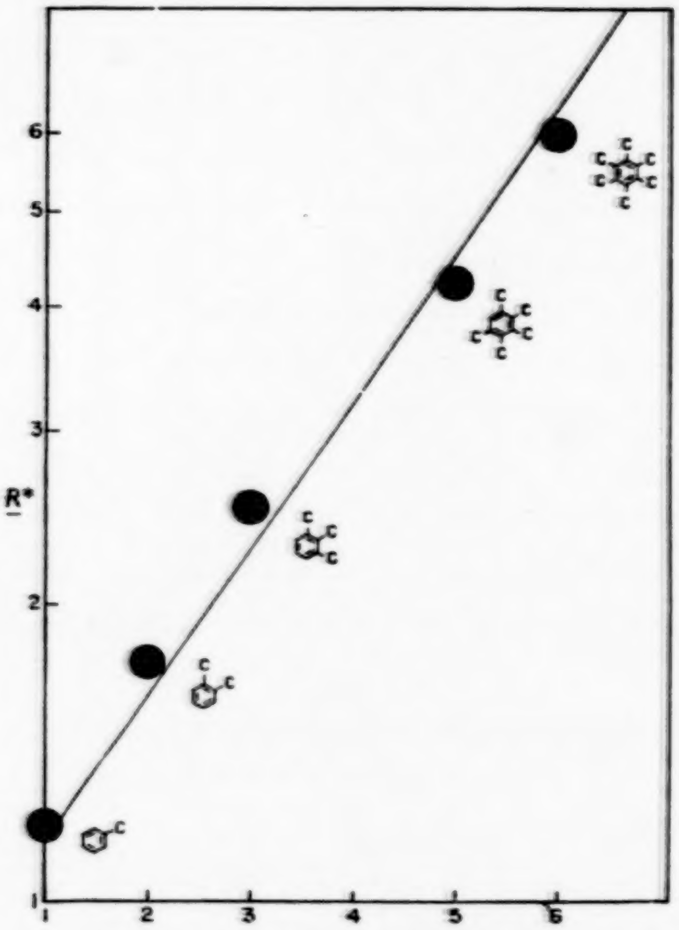


Fig. 11. $\underline{R}^*$ *versus* number of vicinal methyls for substituted benzene; *n*-pentane and 0.5% H_2O–Al_2O_3.

The data of Table VIII further suggest that a naphthene ring has a value of q_j equal to 0.073, apart from the additional contribution of the *ortho* linkage in condensed cycloalkyl aromatics such as tetralin. When the various second-order *and* first-order *alkyl* contributions or values of Q_i and q_j are summed for each solute of Table IV, the *relative* retention volumes can be calculated. These calculated values show an average agreement with experimental of ± 5%; furthermore, those few values which deviate by more than 10% are not suggestive of special effects requiring the postulation of additional second-order terms or q_j values. Consequently it appears reasonable to claim at this point a practical understanding of the effects determining retention volume in the alkyl-substituted aromatic hydrocarbons.

Table IX lists the relative retention volumes for a number of alkylthiophenes. Calculated and experimental $\underline{R}^\circ$ values for the monoalkylthiophenes show good agreement (average deviation $\pm$ 5 %), assuming a value of q_j for any alkyl substitution on the thiophene ring of — 0.036 (*versus* 0.00 for similar substitution on aromatic hydrocarbons). Significant discrepancies arise in the case of the di-substituted compounds, however. The calculated $\underline{R}^\circ$ values for the 2,5-dialkylthiophenes, excluding the iso-butyl derivatives, are consistently high by 20 $\pm$ 4 %. When one of the substituents is iso-butyl, the deviation rises to 29 $\pm$ 1 %, and when both substituents are iso-butyl, the discrepancy is 38 %. These differences between experimental and calculated values obviously parallel the crowding of alkyl groups around the thiophene sulfur, and it is reasonable to postulate that the effect is a result of some sort of interference between the sulfur-adsorbent bond. The value of q_j for alkyl substitution may also reflect this effect. Because the deviations of calculated from experimental values are reasonably constant, it would be possible to derive additional second-order q_j values for specific bonding situations; however, insufficient substitution types are represented in Table IX to generalize on the quantitative dependence of such effects on molecular geometry.

In Table X, the retention volumes of a number of alkyl pyridines and quinolines are presented, for a necessarily less active adsorbent and more polar eluent. For the pyridines which do not possess a 2- or a 6-substituent, and the quinolines not substituted in position 2 or 8, $\underline{R}^\circ$ values can be calculated with rather good overall agreement with experimental data, assuming the q_j values of Table XI for methyl and alkyl substitution on the pyridine and quinoline rings. The one case where the correlation fails is in the case of 3-methyl-4-ethyl-pyridine, and here the disagreement is so large as to cast doubt on the true identity of this commercial sample; there is no reason to invoke a specific second order effect to explain this anomaly. The large value (relative to the hydrocarbons and thiophenes) of q_j for methyl substitution in the nitrogen containing ring (q_j = 0.21) is not unexpected because of the greater basicity of the nitrogen atom, and its related greater sensitivity to electron-donating substituents; this difference should in fact be even larger for more active adsorbents. The reduction of q_j for methyl substitution in the non nitrogen-containing ring of quinoline (q_j = 0.06, relative to the value of 0.21) also emphasizes the greater sensitivity of the nitrogen-containing ring to alkyl substitution. The apparent agreement of this latter quantity with the methyl Q_i of the hydrocarbons is coincidental, since the adsorbent activities are not comparable.

Where one methyl substituent is present in the 2- or 6-position of pyridine, or the 2-position of quinoline, the values calculated using the parameters of Table VI are high by an average of 236 $\pm$ 66 %. This suggests a q_j value of — 0.56 for the calculation of *ortho versus* non-*ortho* methyl pyridines and quinolines. For two such *ortho* methyl groups, the calculated $\underline{R}^\circ$ values are an average of 527 % high, from which a corresponding q_j contribution of — 0.80 can be calculated. These factors emphasize the sensitivity of the nitrogen atom adsorption bond to steric crowding around it. A similar effect has been noted in the case of the *ortho* alkylthiophenes, but there the effect is much smaller, probably due to the smaller energy associated with the sulfur-adsorbent bond. The experimental $\underline{R}^\circ$ value of 8-methylquinoline is substantially smaller than 2-methylquinoline, and this probably reflects more efficient steric interference of a substituent at the 8- as opposed to an *ortho* position.

GLOSSARY OF TERMS

a	subscript refers to adsorbed phase
E	refers to eluent molecule
F_t	free energy per mole of transferring solute from non-sorbed to adsorbed phase (divided by 2.30 RT)
$(F_i)_j$	partial molar free energy of species or group i in phase j (divided by 2.30 RT)
F_{iE}	free energy for transfer of group i from eluent E phase to adsorbed phase (divided by 2.30 RT)
$\underline{K}$	distribution coefficient or equilibrium constant for solute between adsorbed and non-sorbed phases; (g solute per g adsorbent)/(g solute per ml eluent)
$\underline{K}^\circ$	value of K in linear region (concentration-independent value)
K°	thermodynamic equilibrium constant corresponding to $\underline{K}^\circ$; see eqn. (5)
K_{ph}	phenyl group contribution to solute K° value (Sporer and Trueblood[7])
n	subscript refers to non-sorbed phase; also, number of equivalent structural groups; also, number of carbon atoms in alkyl group
Q_i	group i retention volume factor
Q°_i	value of Q_i for calcined alumina
q_j	second-order retention volume factor for geometric arrangement j
q°_j	value of q_j for calcined alumina
r	molal volume ratio of solute to eluent
R'	retention volume; eluent volume required to remove 50% of solute from column
R	corrected retention volume; R' minus V_0 the column void volume
$\underline{R}$	equivalent retention volume, R/W
$\underline{R}^\circ$	equivalent retention volume, R/W, in linear region
$\underline{R}^*$	relative equivalent retention volume, $\underline{R}^\circ$ for substituted aromatic divided by that of unsubstituted compound
V_a	volume of adsorbed phase per gram of adsorbent
V_0	column dead volume in ml
W_s	solute oil to gel ratio; weight of sample divided by weight of adsorbent
x, y, z, i	structural groups which constitute a solute
α	adsorbent activity function
δ_i	relative volume of group i; fraction of eluent molecule displaced from adsorbed phase by i, and varying with eluent
θ	fractional adsorbent loading by solute
θ_i	value of θ for linear elution

ε_{12}	eluent polarity factor, proportional to eluent adsorption energy per unit volume, relative to *n*-pentane
ε°_{12}	value of ε_{12} for calcined alumina
ε°	ε°_{12} with pentane as eluent 1

ACKNOWLEDGEMENT

The author wishes to thank the management of the Union Oil Company of California Research Department for permission to release the present material, his associates, Drs. E. P. PARRY and J. K. FOGO, for conscientious editing and criticism of the original manuscript, Dr. HENRY GILMAN of Iowa State University for the gift of samples of 1-*n*-butyl and 1-*n*-hexyl-naphthalene, the American Petroleum Institute Research Project 42 Group at Pennsylvania State College for the gift of certain other hydrocarbon samples, and the Research Project 48 Group at the United States Bureau of Mines, Laramie, for the donation of the various alkylthiophenes used.

SUMMARY

Linear elution retention volume data have been acquired for alumina as adsorbent. Solute structure, eluent type, and alumina water deactivation have been varied over wide ranges, and the results semiquantitatively correlated in terms of a physical model. Adsorbent deactivation by water is shown to cause progressive reduction in the number of adsorption sites, with the remaining sites being weaker, but partition effects appear unimportant for 0–5 % H_2O–Al_2O_3. Solute adsorption free energy has been found to be a linear function of adsorbent activity. A corollary of this observation is the "constant eluent non-crossing rule": for a given eluent, solute separation order is unchanged as adsorbent activity is varied. For certain chromatographic system and solutes, retention volumes can be quantitatively predicted.

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COLUMN CHROMATOGRAPHY OF *BACTERIUM CADAVERIS* EXTRACTS USING CARBOXYMETHYLCELLULOSE

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(Received October 7th, 1960)

During the past few years the chromatographic separation of proteins on columns of cellulose ion exchangers using gradient elution techniques has won wide approval. However, the lack of standard experimental conditions and of chromatographic characteristics of the separated substances does not often permit comparison of the data of different authors. This paper gives the results of some experiments on the chromatography of proteins on carboxymethylcellulose columns and deals with the possibility of using the elution constant as a new chromatographic characteristic for the various substances.

EXPERIMENTAL AND DISCUSSION

Five protein fractions possessing asparaginase activity were obtained by chromatographing an aqueous extract of *B. cadaveris* on a carboxymethylcellulose (CMC) column. Fraction I consists of a protein that is not adsorbed on CMC from 0.01 M pyridine solution. Fractions II, III and IV were extracted from the CMC column using the gradient elution technique as described by PARR[1] at concentrations of pyridine of 0.023, 0.027 and 0.032 M, respectively. Fraction V was eluted by 0.1 M pyridine or 0.1 M Na_2HPO_4 (Fig. 1). On rechromatography of fraction I under the conditions described, two subfractions were obtained. One of these corresponds to fraction I, the other to fraction V of the initial chromatogram. On rechromatography of fractions V and IV, we obtained in a similar way two and three subfractions respectively. In the case of fraction V, the subfractions correspond to fractions V and IV, in that of fraction IV, to fractions III, IV and V of the initial chromatogram.

Fraction IV contains about 1/10,000 of the total protein applied to the column. The specific activity of asparaginase in this fraction is increased 250-fold as compared with that of the initial *B. cadaveris* acetone powder.

The change in the eluent concentration on elution by the method of PARR can be expressed by the equation

$$C = C_2 - (C_2 - C_1)\left(1 - \frac{v}{V}\right)^{A_2/A_1} \qquad (1)$$

where C is the eluent concentration at the moment when a volume v of the eluate has passed the column; C_1 and C_2 are the concentrations of the solution in the mixing and reservoir chambers; A_1 and A_2 are the cross-sectional areas of the mixing and reservoir chambers; V is their total volume. If $A_1 = A_2$, the equation becomes linear, which is the most favourable case for most separations.

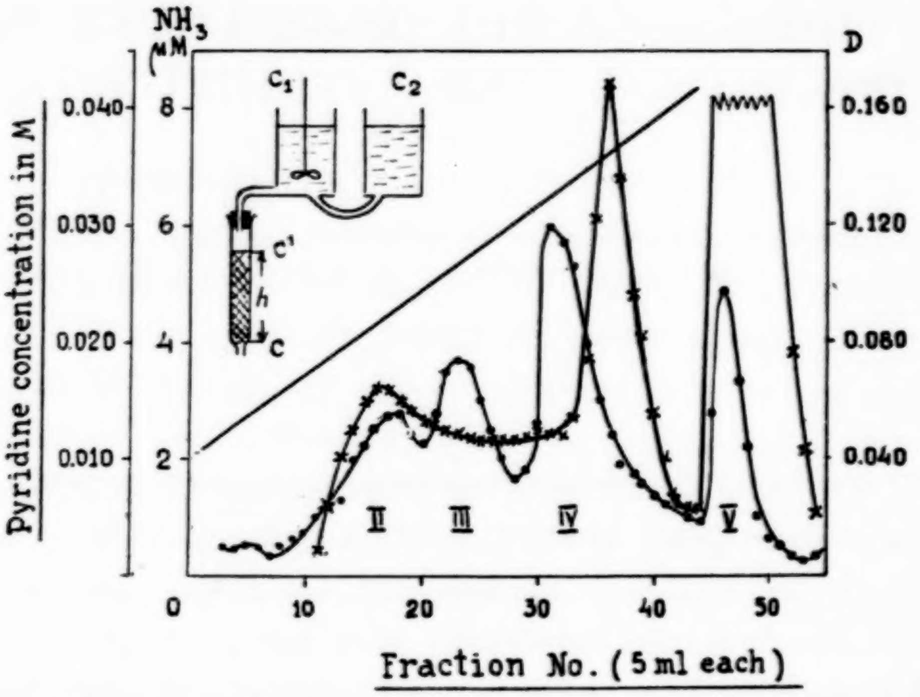

Fig. 1. Column chromatography of *B. cadaveris* extract using CMC. Column size 2.0 × 6.5 cm; $q = 2.5$ ml; washed with 0.010 M pyridine. 25 ml of aqueous extract containing 180 mg protein applied on the column after dialysis against 0.010 M pyridine. Before starting elution by the concentration gradient procedure, the column was washed with 240 ml of 0.010 M pyridine. Elution with aqueous solutions of pyridine: $C_1 = 0.010\ M$, $C_2 = 0.085\ M$, $V = 500$ ml, $G = 3.7 \cdot 10^{-4}$. Asparaginase activity was determined by the method of MARDASHEV AND LESTROVAYA[2], protein as described by LOWRY[3]. (●—●—●) μmoles NH_3; (×—×—×) optical density (D); (————) pyridine concentration.

$$C = C_1 + \frac{v}{V}(C_2 - C_1) \tag{2}$$

However, the separations depend on the actual concentration gradient along the length of the column, which varies not only with C_1, C_2 and V, but also with the cross-section of the column. Therefore, eqn. (2) fails to characterize completely the conditions of separation in gradient elution. To evaluate the concentration gradient along the length of the column we suggest using the value of the change in the concentration of the solution per unit length of the column. If the distance from the base to the top of the column is plotted against the concentration of the solution at various levels, the straight line obtained will represent the change in concentration along the length of the column. The slope of the line determined by angle α expresses the gradient value G

$$G = \operatorname{tg} \alpha = \frac{\Delta C}{h} \tag{3}$$

If a volume of solution v, has passed through the column at a certain moment of time, the concentration at the outflow of the column becomes C; the concentration at a certain distance h from the bottom of the column will at that moment be C'; then

$$G = \frac{\Delta C}{h} = \frac{C' - C}{h} \tag{4}$$

It follows from eqn. (2) that

$$C' = C_1 + \frac{v + v_{bH}}{V} (C_2 - C_1) \tag{5}$$

where v_{bH} is the volume of exchangeable liquid in the pores of a column of length h. Substituting the values of C and C' from eqn. (2) and (5) into eqn. (4), we find:

$$G = \frac{v_{bH}}{h} \cdot \frac{C_2 - C_1}{V} \tag{6}$$

The factor v_{bH}/h is the volume of liquid in the pores of a 1 cm long column, *i.e.*, the "specific pore volume" of the column. Denoting it as q, we get

$$G = q \frac{C_2 - C_1}{V} \tag{7}$$

The value of G represents the actual change in concentration of the eluent along the length of the column. Since q depends on the column radius, the steepness of the gradient change in columns with different radii will be different, other conditions determined by eqn. (2) being constant. Identical conditions of separation are defined by eqns. (8) or (9):

$$G = G' \tag{8}$$

$$q \frac{C_2 - C_1}{V} = q' \frac{C'_2 - C_1}{V'} \tag{9}$$

For $q = 2.5$ [column i.d. (inner diameter) 2 cm], $C_1 = 0.010\ M$, $C_2 = 0.085\ M$ and $V = 500$ ml, the value of G will be:

$$2.5 \frac{0.085 - 0.010}{500} = 3.7 \cdot 10^{-4}$$

In order to have the same gradient operating in a column with $q = 0.6$ ml (column i.d. 1 cm) and the C_1 and V values the same as in the previous experiment, it is necessary to change C_2:

$$C_2 = \frac{VG}{q} + C_1 = \frac{500 \times 3.7 \times 10^{-4}}{0.6} + 0.010 = 0.318$$

If it is required to keep C_1 and C_2 constant, the V value must be changed according to the relationship:

$$\frac{q}{V} = \frac{q'}{V'}$$

which is derived from eqn. (9) if $C_2 = C_2'$.

With such a characteristic of the gradient, the relative distances between the peaks on the chromatogram of a certain mixture will remain the same, independent of the dimensions of the column used.

It can be shown that:

$$\frac{v}{q} = \frac{v'}{q'} = \text{const.} = K_e \qquad (10)$$

where v and v' are the volumes of solutions that have passed through columns with the specific pore volumes q and q' up to the moment when the concentration C in both effluents becomes equal, under the condition that $G = G'$. Therefore, the ratio of the volume of effluent collected from the beginning of the elution to the moment of appearance of the maximal concentration of the given substance, to the specific pore volume of the column—the elution constant K_e—may be considered as an accurate chromatographic constant of a substance and may be used for purposes of identification. From certain theoretical considerations and experimental data[4,5] it follows that in gradient elution the rate of migration of the zone of a substance tends to attain a maximum equal to the rate of flow of the solvent itself. This means that K_e is independent of the column length, provided the length is not less than that necessary for attaining maximum velocity (and simultaneously best separation) for the concentration gradient selected.

The K_e values for the three asparaginase peaks at $G = 3.7 \cdot 10^{-4}$ obtained in columns of various sizes are given in Table I.

TABLE I

Column size cm	q	K_e		
		II	*III*	*IV*
2.0 × 6.5*	2.5	36.0	46.0	60.0
1.2 × 10	0.9	37.7	46.4	63.3
1.0 × 16	0.6	38.2	48.3	66.6

* The first figure represents the inner diameter, the second the length.

SUMMARY

1. A method is suggested for the evaluation of the changes in concentration of an eluent along the length of a column in gradient elution chromatography.

2. A new chromatographic constant—the elution constant K_e—is proposed for substances being chromatographed. K_e is defined as the ratio of the effluent volume, collected up to the moment of appearance of the maximal concentration of the substance being chromatographed, to the specific pore volume of the column.

3. The elution constants have been determined for three protein fractions of a *B. cadaveris* extract possessing asparaginase activity.

4. By means of column chromatography using carboxymethylcellulose, it was possible to attain a 250-fold purification of asparaginase.

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A CHROMATOGRAPHIC FRACTION-SAMPLING DEVICE

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(Received October 14th, 1960)

In the preparative scale separation of amino acids and peptides, by the methods of HIRS, MOORE AND STEIN[1-3], the usual method for the location of peaks has been to take samples from the effluent fractions, and subject these to reaction with ninhydrin, either before or after alkaline hydrolysis. Since analysis of amino acids can now be done automatically[4-7], it is desirable also to mechanize preparative separations.

Using the apparatus of SIMMONDS[5], the device described below was devised to sample each fraction automatically, diverting the sample to the analysis machine, while the remainder of the fraction was collected separately. It is, in fact, more general in its application, and can be used whenever effluent fractions need to be divided into two portions, for subsequent analysis.

DESCRIPTION OF APPARATUS

The fraction-sampling device is shown in Fig. 1. When the body of the collecting tube is full, liquid escapes via the overflow B into a drop-counting chamber. When the required number of drops has been collected for the sample, the valve V is lifted by the solenoid S, and the main part of the fraction is delivered to the fraction collector.

The fraction volume can be adjusted by inserting extension pieces between the upper and lower parts of the collecting chamber. In this way, the volume delivered can be easily varied between say 2 ml and 20–30 ml, by the use of a graduated series of extension pieces. Preferably, the apparatus can be scaled up for larger fractions.

The sample volume can be determined in any convenient way. A conductivity-operated, electromechanical drop counter* has been used, but any other drop counter or volume-operated device (see *e.g.*, SIMMONDS[5]) can be used. The circuit used to lift the valve, V, and to operate the turntable is as used by SIMMONDS[5], except that the input terminals are bridged by the drop counter.

The sample may be diverted into a second row of tubes in the fraction collector, or into any suitable automatic recording device. When used with the apparatus of SIMMONDS[5] for amino acid or peptide separations, the electrical arrangement is as shown in Fig. 2. The recording machine is started by an extra set of contacts on the relay controlling valve V (Fig. 1). If required, a short alkaline hydrolysis can be done before the ninhydrin reaction, in an additional heating vessel mounted above the

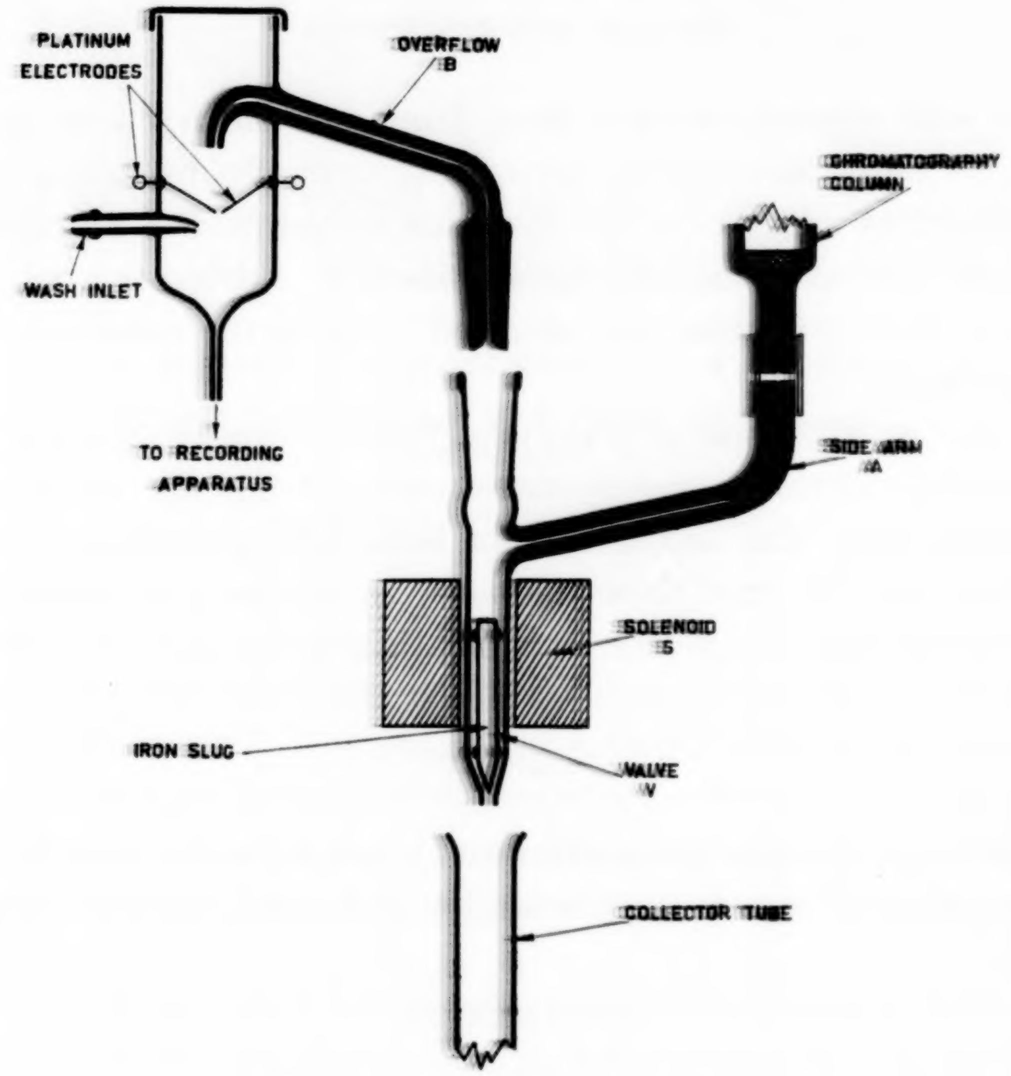
PLATINUM ELECTRODES
OVERFLOW B
CHROMATOGRAPHY COLUMN
WASH INLET
TO RECORDING APPARATUS
SIDE ARM A
SOLENOID S
IRON SLUG
VALVE V
COLLECTOR TUBE

Fig. 1. Fraction-sampling device.

ninhydrin heating vessel, and controlled by an additional bank of motor-operated switches.

When working with small samples, it may be desirable to wash the sample into the recorder by means of a jet of distilled water (or other suitable liquid) from a dispenser of the type used by SIMMONDS[5], and operated in parallel with the solenoid S (Fig. 1).

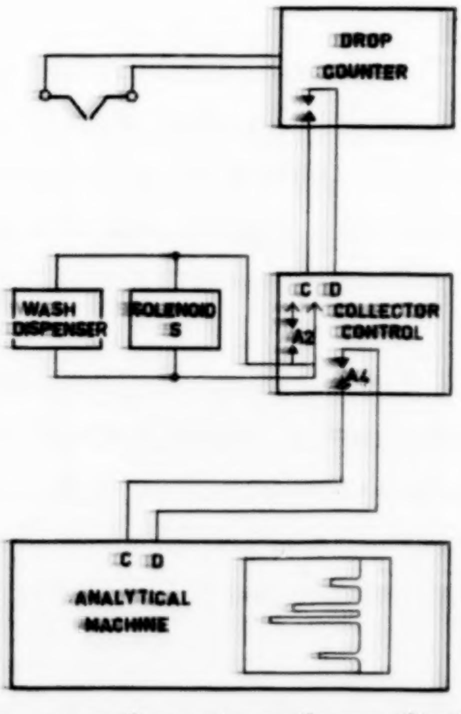
DROP COUNTER
WASH DISPENSER
SOLENOID S
C D
COLLECTOR CONTROL
A2
A4
C D
ANALYTICAL MACHINE

Fig. 2. Electrical arrangement for automatic monitoring of column effluent.

RESULTS AND DISCUSSION

Various amino acid separations have been done, and the fractions analysed both automatically, by using the sampling device, and manually, by taking aliquots from the main fractions, as a check on the operation of the device. Fraction sizes have been varied from 3 ml to 10 ml, and sample size from 2 drops to 2 ml. For all such combinations, a close correlation was obtained between the automatic and manual absorbance readings.

For most purposes location of peaks is all that is required, but if a quantitative estimation is needed, this can be done by measurement of drop size, and standardization in the appropriate way. This aspect has not been fully investigated, but provided peaks are spread over at least three fractions, an accuracy of better than ± 8% appears to be obtainable, despite the fact that there is no provision for mixing.

SUMMARY

A fraction-collecting apparatus is described which automatically samples each fraction in turn as it is collected, and diverts the sample for analysis by any suitable method.

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EINE SYSTEMATISCHE ANALYSE VON ALKALOIDEN AUF DÜNNSCHICHTPLATTEN

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(Deutschland)

(Eingegangen den 27. Oktober 1960)

In einer früheren Arbeit[1] haben wir die Alkaloide auf Grund ihres polaren Verhaltens in einem System: Formamid/Cyclohexan–Chloroform–Diäthylamin, papierchromatographisch in 4 verschiedene Gruppen einteilen können. Nach Ermittlung der Gruppenzugehörigkeit in einem kurzen Vorversuch konnten dann die einzelnen Alkaloide durch ihr unterschiedliches Verhalten in Fliessmitteln wechselnder Zusammensetzung identifiziert werden.

In der vorliegenden Arbeit werden die Alkaloide in analoger Weise nach ihrem Verhalten auf Dünnschichtplatten in zwei Gruppen unterteilt. Aus der Lage der gefundenen Flecke und unter eventueller Zuhilfenahme weiterer angegebener Lösungsmittelkombinationen lassen sich die Alkaloide identifizieren. Es wird ferner das Verhalten der Alkaloide auf verschiedenen Trägerschichten (Kieselgel sauer, Aluminiumoxyd basisch) besprochen.

Die neue Dünnschichtchromatographie nach STAHL[2-7] ist besonders gut zur Entwicklung jener Verbindungen geeignet, die bisher papierchromatographisch auf üblicher Weise formamidimprägnierten Filtrierpapieren getrennt wurden. So konnten von uns bereits verschiedene Steroide[8] vorteilhaft auf Dünnschichtplatten chromatographiert werden.

Die Bedeutung der Dünnschichtchromatographie[**] ist unverkennbar durch ihre Vorzüge gegenüber der Papierchromatographie gegeben. Diese sind unter anderen: die kurze Entwicklungszeit (etwa 20–40 Minuten), die gute Anfärbbarkeit und Sichtbarmachung auf einem anorganischen Träger (die Empfindlichkeit der Anfärbung ist meist eine oder zwei Zehnerpotenzen grösser als auf Papier) und die runden, sauberen Fleckenformen.

Eine Trennung mehr oder weniger hydrophober Verbindungen auf aktivierten Platten geht mit vorwiegend hydrophoben Lösungsmitteln (in der Hecker'schen Reihe von Chloroform bis zu den Kohlenwasserstoffen) adsorptionschromatographisch vor sich. Es ist möglich, beim Übergang zu hydrophileren Verbindungen auf Platten in analoger Weise wie auf dem Filtrierpapier eine Trennung durch Verteilung zu erreichen, wobei dann eine oder mehrere stark wasserlösliche Komponenten am Aufbau des Fliessmittels beteiligt sind. Diese setzen dann allerdings die Aktivität der Platten herab, weil sie an die Adsorptionszentren gebunden werden.

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[*] Leiter: Prof. Dr. K. KREBS.

[**] Prospekt: Aluminiumoxyd G, Kieselgel G, Kieselgur G für die Dünnschichtchromatographie kann bei der Fa. E. Merck AG, Darmstadt, angefordert werden.

Das der Grundausrüstung* für die Dünnschichtchromatographie beiliegende Testgemisch, gibt ein Mass für die relative Aktivität des Trägermaterials. Wie aus Fig. 1 ersichtlich, hat Kieselgel G die grösste Aktivität, dann folgen Aluminiumoxyd G und schliesslich die praktisch inaktive Kieselgur G. Auf allen drei Platten wurde das Testgemisch mit Benzol entwickelt. Je grösser die Aktivität ist, um so kleiner sind die R_F-Werte.

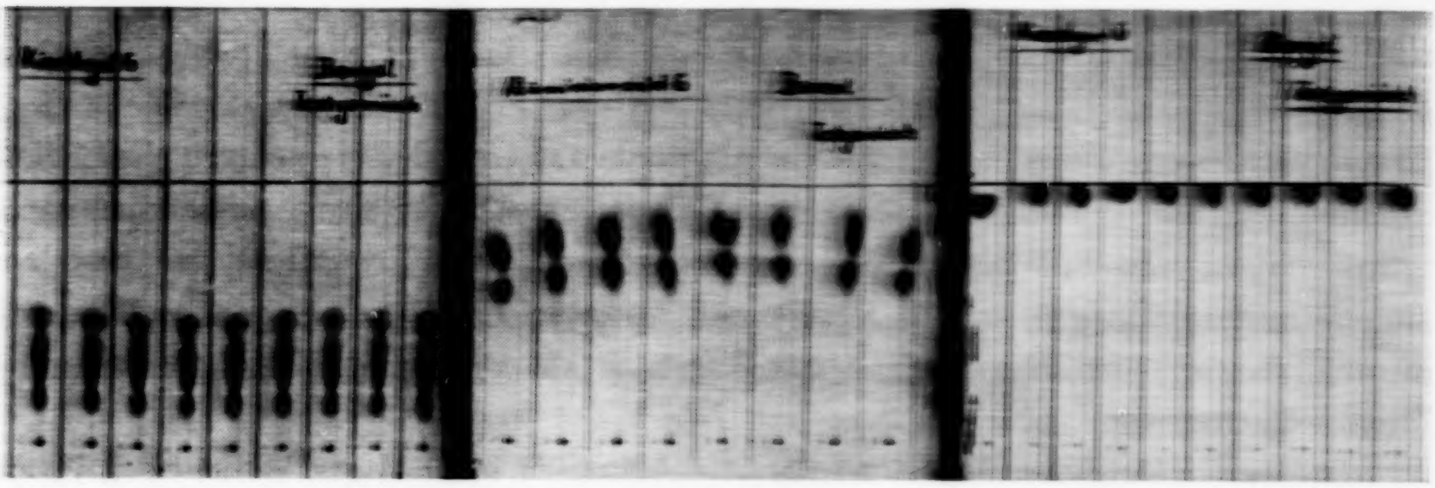

Fig. 1. Verhalten von drei Farbstoffen (Testgemisch) auf verschiedenen Schichten mit Benzol. Kieselgel G, Aluminiumoxyd G und Kieselgur G.

Mit der Aktivität parallel geht die Kapazität des Trägers, die sich auf die maximale Auftragsmenge bezieht, welche noch ohne Schwanzbildung (tailing) zu entwickeln ist. Auf Kieselgelplatten betragen diese Substanzmengen bis zu einigen Milligramm, auf Aluminiumoxyd bis zu wenigen Zehntelmilligramm und auf inaktiven Kieselgurplatten im allgemeinen nur 25–50 µg.

Bei der Entwicklung von Verbindungen, die saure oder basische Gruppen enthalten, müssen entsprechende Trägereigenschaften ebenfalls berücksichtigt werden. So reagieren die Kieselgel G-Platten stark sauer, und Basen werden mit neutralen Lösungsmitteln am Startpunkt festgehalten. Aluminiumoxyd G-Platten reagieren basisch und binden saure Komponenten, Kieselgur G-Platten verhalten sich neutral.

Es ist interessant, dass sich das mit Formamid imprägnierte Filtrierpapier Basen gegenüber ähnlich wie Kieselgel G-Platten verhält. Mit neutralen Lösungsmitteln, z.B. Chloroform, verbleiben Basen bei der Entwicklung auf dem Startpunkt.**

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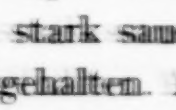
* Zu beziehen bei Desaga GmbH, Heidelberg, Hauptstr. 60.

** Wir nehmen an, dass Formamid – das an das Papier gebunden ist – Basen gegenüber in der sauren Enolform reagiert:



$$\underset{\displaystyle OH}{\underset{|}{HC}}=NH$$

Formamid für die Chromatographie Merck zeigt mit der Messelektrode schwach basische Reaktion (pH 7.5–8.5). Verschiedentlich wurde beobachtet, dass Formamid anderer Provenienzen mit schwach saurem Verhalten zur Entwicklung der Alkaloide nach unserem Schema[1] praktisch unbrauchbar ist, da die R_F-Werte viel zu tief liegen. Ein Zusatz von Ammoniumformiat half meist auch nicht[9–11].

Die als freie Basen vorliegenden Alkaloide können mit einfachen Lösungsmitteln, z.B. Chloroform, auf basischen Aluminiumoxyd G-Platten ohne weiteres adsorptionschromatographisch entwickelt und teilweise auch getrennt werden, wie Fig. 2(a) zeigt. Da die Basenkapazität solcher Platten verhältnismässig gering ist, tritt bei etwas höheren Auftragsmengen (über 50 μg) teilweise Schwanzbildung ein. Durch den geringfügigen Zusatz einer starken, flüchtigen Base zum Elutionsmittel ist es möglich, die Auftragsmengen auf diesen Platten zu erhöhen. Allerdings wird bei Zugabe von nur 0.05% Diäthylamin (3 Tropfen) die Aktivität der Aluminiumoxydplatte herabgesetzt, was durch Einsetzen eines weiteren, hydrophoberen Lösungs-

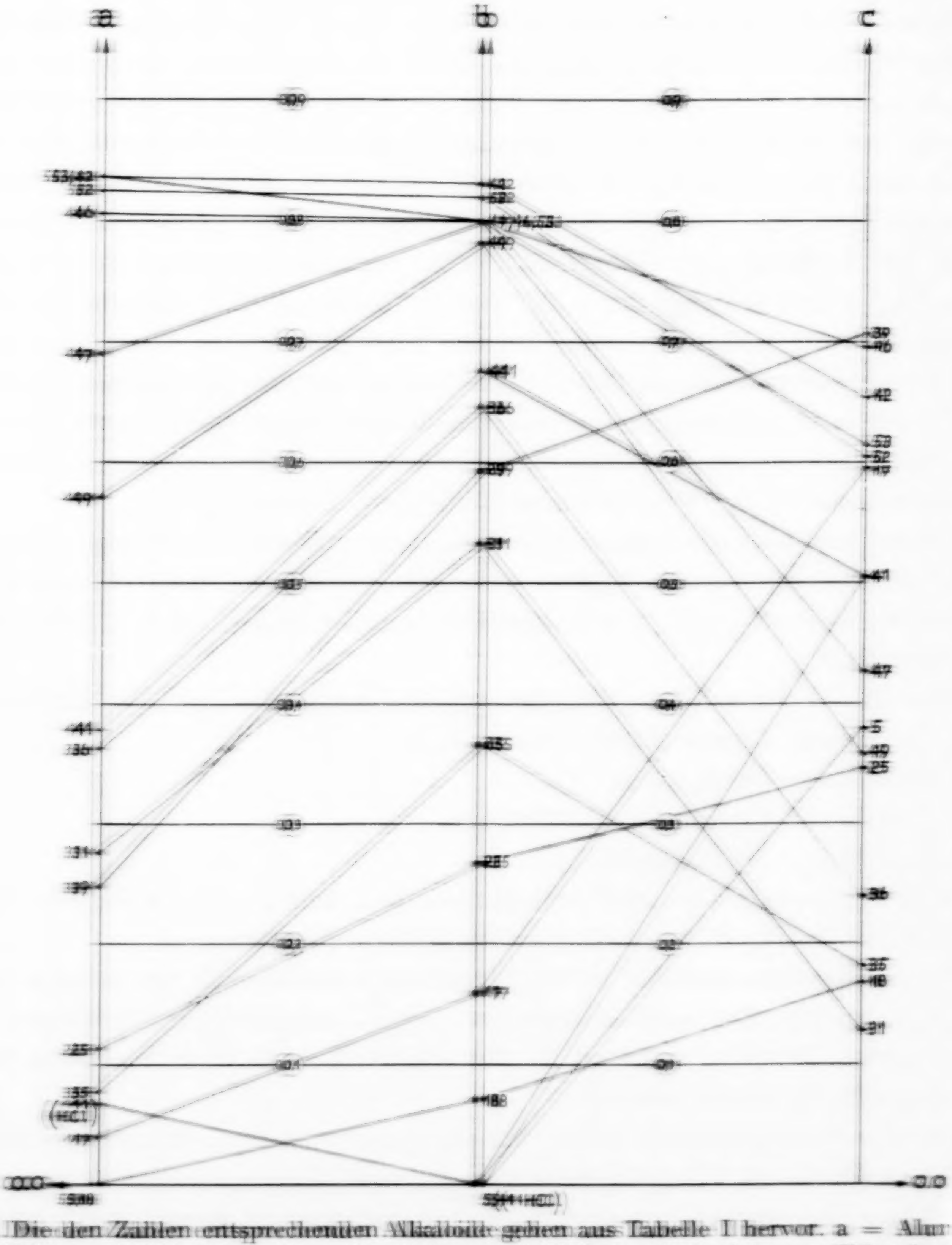


Fig. 2. Die den Zahlen entsprechenden Alkaloide gehen aus Tabelle I hervor. a = Aluminiumoxyd G-Platte mit Chloroform; b = Aluminiumoxyd G-Platte mit Cyclohexan–Chloroform (3:7) 0.05% Diäthylaminzusatz; und c = Kieselgelplatte, mit N/10 Natronlauge hergestellt und mit Methanol entwickelt.

mittels (Cyclohexan) ausgeglichen wird (Fig. 2 (b)). Dieser geringfügige Zusatz von Diäthylamin reicht allerdings nicht aus, um die Basen vollständig aus ihren Salzen frei zu machen. So verbleibt beispielsweise Emetinhydrochlorid (Nr. 41 HCl der Fig. 2 (a und b)) praktisch am Startpunkt, und nur ein kleiner Anteil wird während der Entwicklung als Base frei, so dass ein gezogener Fleck entsteht. Im allgemeinen sind die R_F-Werte der Basen auf Aluminiumoxydplatten nicht so gut reproduzierbar wie auf Kieselgelplatten.

Es gibt verschiedene Möglichkeiten, mehr oder weniger stark basische Alkaloide auch auf Kieselgel G-Platten zur Entwicklung zu bringen. Die sauren Gruppen des Kieselgels müssen zuvor gebunden werden. STAHL[7] hat die schwach basischen Mutterkornalkaloide auf den sauren Kieselgelplatten durch Verteilung mit Chloroform–Methanol trennen können. In der gleichen Arbeit berichtet dieser Autor über die Anfertigung basischer Kieselgelschichten. Zum Herstellen solcher Platten wird Kieselgel G anstelle von Wasser mit N/10 Natronlauge angerührt. Auf diesen Platten lassen sich die Alkaloide mit Methanol entwickeln. Die als Salze aufgetragenen Alkaloide werden restlos in die Basen übergeführt und ergeben meist schöne, runde Fleckenformen. Die Umkehr verschiedener R_F-Werte einzelner Alkaloide (vergleiche z.B. Brucin Nr. 31 und Morphin Nr. 5 der Fig. 2 (b und c)) lässt auf einen ähnlichen Trennungsvorgang schliessen, wie es bei der Entwicklung von Basen oder Säuren (Phenole) mit Elektrolytlösungen auf Filtrierpapier bereits vorgenommen wurde[12,13].

Auf den oben beschriebenen alkalischen Platten (Fig. 2 (a–c)) lassen sich sämtliche Alkaloide mit einem Lösungsmittel oder Lösungsmittelgemisch jeweils auf *einer* Platte entwickeln (siehe hierzu auch Tabelle I, Entwickler a–c).

Um auf den üblichen sauren Kieselgelplatten Alkaloide in analoger Weise wie auf formamidimprägniertem Papier entwickeln zu können, wird der Steiglösung eine stärkere Base zugesetzt. Wir fanden auch hier den Zusatz von 10 % Diäthylamin recht vorteilhaft.

Über das Verhalten der Alkaloide auf Kieselgelplatten mit den Entwicklern:

1. Chloroform–Aceton–Diäthylamin (5:4:1)
2. Chloroform–Diäthylamin (9:1)
3. Cyclohexan–Chloroform–Diäthylamin (5:4:1)
4. Cyclohexan–Diäthylamin (9:1)

vermittelt Fig. 3 einen Überblick. Von Entwickler 2 nach 4 ist ein mehr oder weniger grosser Abfall der R_F-Werte sämtlicher Alkaloide festzustellen.

Wie bereits oben erwähnt, ist bei Entwickler 2 eine Grenze von Adsorption und Verteilung erreicht. Die meisten Alkaloide zeigen bezüglich ihres R_F-Wertes von 2 nach 1 fallende Tendenz, während die Mutterkornalkaloide (auch Yohimbin Nr. 30) noch steigende R_F-Werte aufweisen.

Auf den Kieselgelplatten lassen sich die 54 untersuchten Alkaloide weiter auseinanderziehen als auf basischen Schichten. So wird die erste Gruppe (1–37) nur mit Entwickler 2, die zweite Gruppe (38–54) im wesentlichen mit Entwickler 3 chromatographiert.

Wie aus Tabelle I ersichtlich, trennen sich die Alkaloide 1–37 in Entwickler 2,

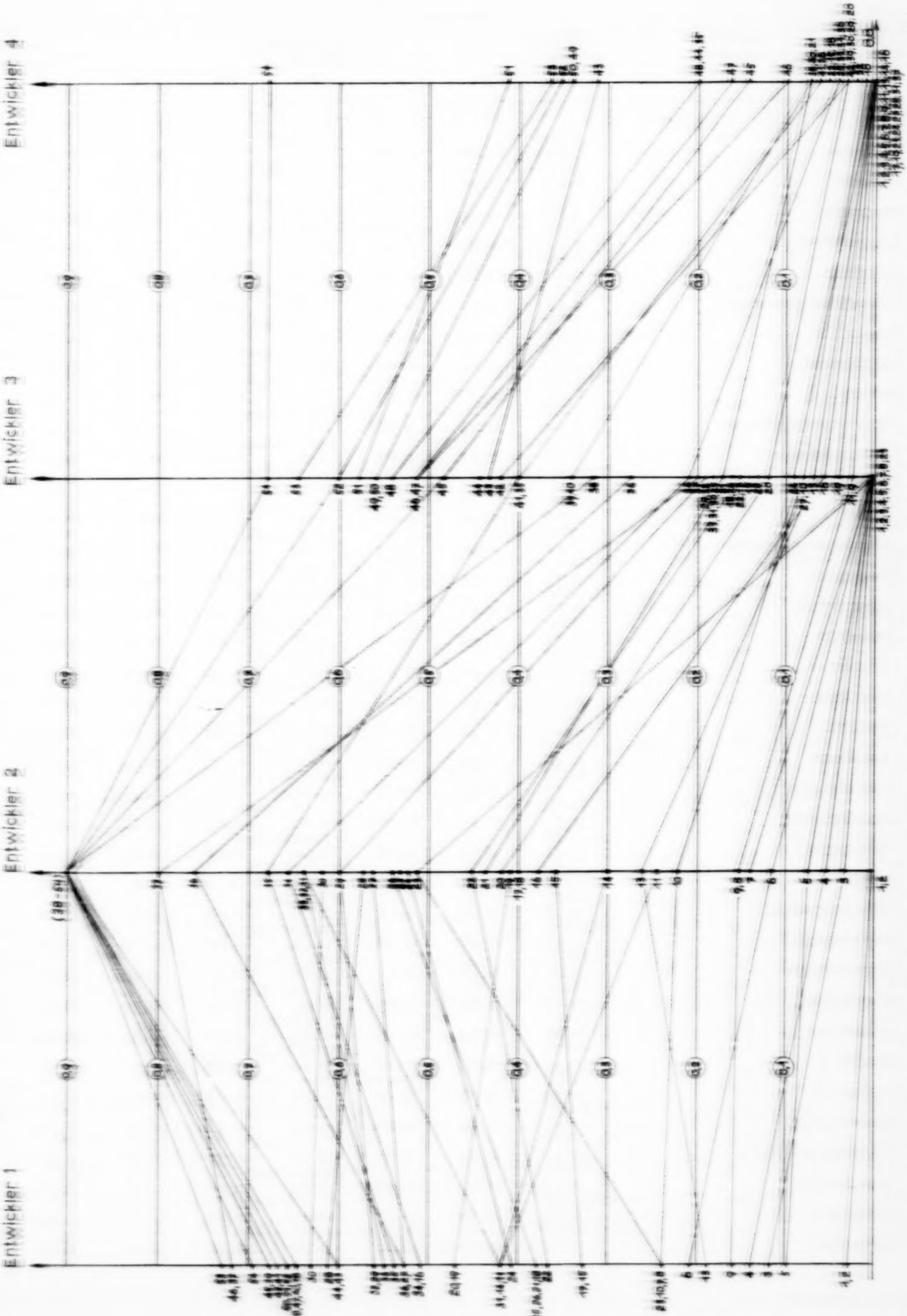


Fig. 3. Kieselgel G-Platten mit: E-1 = Chloroform–Aceton–Diäthylamin (5:4:1); E-2 = Chloroform–Diäthylamin (9:1); E-3 = Cyclohexan–Chloroform–Diäthylamin (5:4:1); E-4 = Cyclohexan–Diäthylamin (9:1) entwickelt. Die den Zahlen entsprechenden Alkaloide sind der Tabelle I zu entnehmen.

TABELLE

Gruppe II

Nr.	Alkaloid	Rf-Werte in							
		E-1	*E-2*	*E-3*	*E-4*	*E-5*	*a*	*b*	*c*
1	Narcein	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	Cuprein	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.46
3	Sinapin	0.12	0.04	0.00	0.00	0.00	0.00	0.00	0.00
4	Ergometrin	0.14	0.06	0.00	0.00	0.02	0.03	0.00	0.64
5	Morphin	0.10	0.08	0.00	0.00	0.03	0.03	0.00	0.34
6	Dihydroergotamin	0.21	0.12	0.00	0.00	0.03	0.07	0.00	0.61
7	Serpentin	0.24	0.15	0.00	0.00	0.04	0.00	0.00	0.00
8	Ergotamin	0.24	0.16	0.00	0.00	0.03	0.10	0.05	0.59
9	Boldin	0.16	0.16	0.03	0.00	0.05	0.24	0.06	0.58
10	Dihydromorphinon	0.24	0.23	0.08	0.01	0.11	0.05	0.08	0.16
11	Ergometrinin	0.42	0.25	0.03	0.00	0.08	0.12	0.10	0.62
12	Ephedrin	—	—	—	—	—	—	—	—
13	Chinin	0.19	0.26	0.07	0.00	0.17	0.09	0.18	0.43
14	Dihydroergocristin	0.42	0.30	0.03	0.00	0.07	0.15	0.07	0.69
15	Hordenin	0.33	0.36	0.14	0.05	0.28	0.00	0.15	0.35
16	Ergocristin	0.51	0.38	0.14	0.05	0.13	0.46	0.15	0.70
17	Chinidin	0.33	0.40	0.15	0.00	0.25	0.12	0.18	0.50
18	Atropin	0.38	0.40	0.16	0.05	0.12	0.00	0.10	0.17
19	Colchicin	0.47	0.41	0.04	0.00	0.04	0.11	0.00	0.57
20	Ajmalin	0.47	0.42	0.12	0.03	0.30	0.06	0.13	0.56
21	Cinchonin	0.38	0.44	0.17	0.07	0.27	0.00	0.22	0.40
22	Homatropin	0.37	0.45	0.15	0.05	0.23	0.04	0.24	0.15
23	Ergotaminin	0.24	0.51	0.00	0.00	0.14	0.42	0.15	0.68
24	Pilocarpin	0.41	0.52	0.09	0.00	0.13	0.32	0.25	0.55
25	Codein	0.38	0.53	0.16	0.04	0.26	0.12	0.27	0.35
26	Dihydrocodein	0.38	0.54	0.18	0.06	0.28	0.10	0.30	0.25
27	Serpentinin	0.53	0.56	0.08	0.00	0.10	0.00	0.03	0.12
28	Ergocristinin	0.61	0.57	0.13	0.00	0.20	0.00	0.27	0.70
29	Scopolamin	0.56	0.60	0.19	0.03	0.34	0.30	0.00	0.52
30	Yohimbin	0.63	0.62	0.18	0.03	0.37	0.33	0.15	0.60
31	Brucin	0.42	0.63	0.18	0.00	0.19	0.50	0.54	0.12
32	Cephaelin	0.56	0.63	0.19	0.02	0.23	0.25	0.17	0.37
33	Rauwolscin	0.55	0.63	0.18	0.04	0.36	0.36	0.15	0.68
34	Dihydrocodeinon	0.51	0.65	0.21	0.04	0.30	0.48	0.43	0.18
35	Apoatropin	0.54	0.67	0.40	0.20	0.26	0.15	0.40	0.16
36	Strychnin	0.53	0.76	0.28	0.05	0.38	0.57	0.60	0.22
37	Reserpin	0.72	0.80	0.20	0.00	0.46	0.63	0.35	0.69

I

Gruppe I

Fluoreszenzfarbe im U.V. (365 mμ)	*Farbe nach Besprühen mit Jodplatinat Rg.*	*Gefundene Fleckenform*	*Zahl der Nebenflecke*	*Zahlenfolge mit PC*
—	tiefblau	rund	—	2
bräunlich-gelb	rotbraun	rund	—	5
—	beige	rund	—	1
violettblau	weiss (rosa Untergr.)	rund	—	3
—	tiefblau	rund	—	4
violettblau	bräunlich	rund	—	13
dunkelbraun	rotbraun	rund	—	7
violettblau	rosa	rund	—	14
violett	beige	rund	—	9
—	bräunlich-gelb	rund	—	8
violettblau	violettblau	rund	—	11
—	hellbraun	gezogen!!!	—	43
blau	gelbweiss	rund	—	41
violettblau	bräunlich	rund	—	18
—	weiss (rosa Untergr.)	rund	—	36
violettblau	beige-hellbraun	rund	—	16
blau	hellgelb	rund	1	44
—	violettblau	rund	—	37
—	hellgrau	rund	—	12
bläulich	beige	rund	2	33
—	beige-braun	rund	—	45
—	violettblau	rund	—	35
violettblau	rosa	rund	—	15
—	hellbraun	rund	—	6
—	ericafarben	rund	—	30
blau	violettblau	rund	—	32
gelbgrün	gelbbraun	gezogen	—	19
violettblau	hellbraun	rund	—	17
—	violett	rund	—	28
grünblau	hellgelb	rund	3	31
—	violettbraun	rund	1	20
violettblau	weiss (rosa Untergr.)	rund	3	22
gelbgrün	schwach beige	rund	1	42
—	violett	rund	2	25
—	violettblau	rund	—	52
—	gelb	gezogen	—	24
grüngelb	weiss (rosa Untergr.)	rund	3	34

(*Fortsetzung auf Seite 68*)

TABELLE I

Gruppe II

Nr.	Alkaloid	R_F-Werte in *E-1*	*E-2*	*E-3*	*E-4*	*E-5*	*a*	*b*	*c*
38	Physostigmin	0.65	> 0.9	0.32	0.04	0.44	0.59	0.50	0.46
39	Aconitin	0.68	> 0.9	0.35	0.03	0.49	0.36	0.60	0.65
40	Bulbocapnin	0.65	> 0.9	0.35	0.07	0.54	0.78	0.70	0.48
41	Emetin	0.67	> 0.9	0.40	0.06	0.45	0.38	0.58	0.50
42	Papaverin	0.67	> 0.9	0.42	0.03	0.47	0.85	0.84	0.70
43	Cotarnin	0.60	> 0.9	0.43	0.31	0.45	0.00	0.25	0.00
44	Scopolin	0.60	> 0.9	0.44	0.20	0.44	0.46	0.50	0.37
45	Lobelin	0.68	> 0.9	0.48	0.14	0.48	0.55	0.60	0.55
46	Narcotin	0.72	> 0.9	0.51	0.10	0.57	0.81	0.79	0.72
47	Thebain	0.65	> 0.9	0.51	0.16	0.50	0.71	0.76	0.40
48	Aspidospermin	0.65	> 0.9	0.54	0.20	0.49	0.50	0.60	0.65
49	Tropacocain	0.65	> 0.9	0.56	0.34	0.45	0.58	0.78	0.35
50	Arecolin	0.66	> 0.9	0.56	0.34	0.48	0.00	0.00	0.00
51	Hydrastinin	0.66	> 0.9	0.58	0.41	0.50	0.00	0.25	0.00
52	Psicain neu	0.66	> 0.9	0.60	0.35	0.53	0.83	0.82	0.59
53	Cocain	0.73	> 0.9	0.65	0.36	0.58	0.84	0.77	0.62
54	Spartein	0.70	> 0.9	0.68	0.68	0.55	0.00	0.55	0.05

Die E-Systeme beziehen sich auf Kieselgelplatten nach STAHL, während a, b und c entsprechend der Fig. 2 auf alkalischen Schichten auszuführen ist, und zwar a und b auf Aluminiumoxyd, c auf N/10 NaOH-vorbehandelten Kieselgelplatten. Die Eluentien (Entwickler) mit entsprechendem Mittelwert des Farbvergleichs sind:

Bezeichnung	*Entwickler*	*Farbvergleich*	
E-1	Chloroform–Aceton–Diäthylamin (5:4:1)	Rhodamin B	R_F 0.58
E-2	Chloroform–Diäthylamin (9:1)	Rhodamin B	R_F 0.49
E-3	Cyclohexan–Chloroform–Diäthylamin (5:4:1)	Rhodamin B	R_F 0.20
E-4	Cyclohexan–Diäthylamin (9:1)	Buttergelb	R_F 0.45
E-5	Benzol–Äthylacetat–Diäthylamin (7:2:1)	Buttergelb	R_F 0.44
a	Chloroform	Buttergelb	R_F 0.85
b	Cyclohexan–Chloroform (3:7) + 0.05% Diäthylamin (3 Tropfen)	Buttergelb	R_F 0.85
c	Methanol	Rhodamin B	R_F 0.53

Die Zahlenfolge bezieht sich von 1–37 auf E-2, von 38–54 auf E-3.
PC = Papierchromatographie.

während die übrigen mit einem R_F-Wert über 0.9 wandern. Die Alkaloide 38–54 entwickeln sich in Entwickler 3 (E-3) über R_F 0.3, während sich die der ersten Gruppe im unteren Bereich befinden. Die R_F-Werte der Tabelle I sind auf einen mitgewanderten Farbstoff korrigierte Mittelwerte aus mehreren Bestimmungen. Die Zahlenfolge der Alkaloide in den Entwicklern 2 und 3 wurde zusätzlich auf den Platten überprüft. Die

(*Fortsetzung*)

Gruppe II				
Fluoreszenzfarbe im U.V. (365 mμ)	*Farbe nach Besprühen mit Jodplateat Rg.*	*Gefundene Fleckenform*	*Zahl der Nebenflecke*	*Zahlenfolge mit PC*
—	rosa	rund	1	23
—	rotbraun	rund	3	46
blau	weiss (rosa Untergr.)	rund	—	29
blau	rotbraun	rund	3	39
gelblich	gelb	rund	—	26
grüngelb	violett	gezogen	1	21
—	weiss (rosa Untergr.)	rund	—	27
—	rotbraun	rund	—	47
blau	hellgelb	rund	—	40
—	rotbraun	rund	—	38
—	weiss (rosa Untergr.)	rund	—	49
—	violett	rund	—	50
—	weiss (rosa Untergr.)	rund	—	—
stahlblau	violettblau	gezogen	1	10
—	gelb	rund	—	51
—	violett	rund	—	48
—	violett	rund	—	54

Entwickler 1, 4 und 5 dienen zur weiteren Differenzierung besonders der nahe beieinanderliegenden Alkaloide, die mit den untersuchten Eluentien andere Löslichkeitseigenschaften aufweisen und deshalb oft die Reihenfolge wechseln.

Die nach der Entwicklung erhaltenen Flecke sind meist rund. Einige Alkaloide zeigen Nebenflecke, die in einer besonderen Rubrik der Tabelle I aufgeführt sind. Ephedrin (Nr. 12) lässt sich auf Filtrierpapier einwandfrei entwickeln. Auf Platten stellen wir bei diesem Alkaloid meist gezogene Flecken fest, was auf Zersetzung deutet. Auch mit Aceton–Methanol–Eisessig (5:4:1) sind auf Kieselgelplatten noch gezogene Flecke feststellbar. Der R_F-Wert des Ephedrins lässt sich mit 0.32, bezogen auf Rhodamin B (R_F 0.62), ermitteln. Die Sichtbarmachung erfolgt mit Cersulfat-Reagenz nach Sonnenschein.

DIE AUSFÜHRUNG EINER ALKALOIDANALYSE AUF KIESELGELPLATTEN

Mit Hilfe von Tabelle I bzw. Fig. 3 ist es möglich, eine praktische Alkaloidanalyse durchzuführen. Wir verweisen auf den experimentellen Teil am Schluss dieser Arbeit.

Die zu untersuchenden Alkaloide sollen gelöst in einem Konzentrationsbereich von etwa 0.05–5 % vorliegen. Salze der Alkaloide werden zu Beginn der Entwicklung durch den Diäthylaminzusatz in die Basen überführt. Mit der ersten Kieselgelplatte werden die günstigste Auftragekonzentration und die Gruppenzugehörigkeit des gesuchten Alkaloids ermittelt.

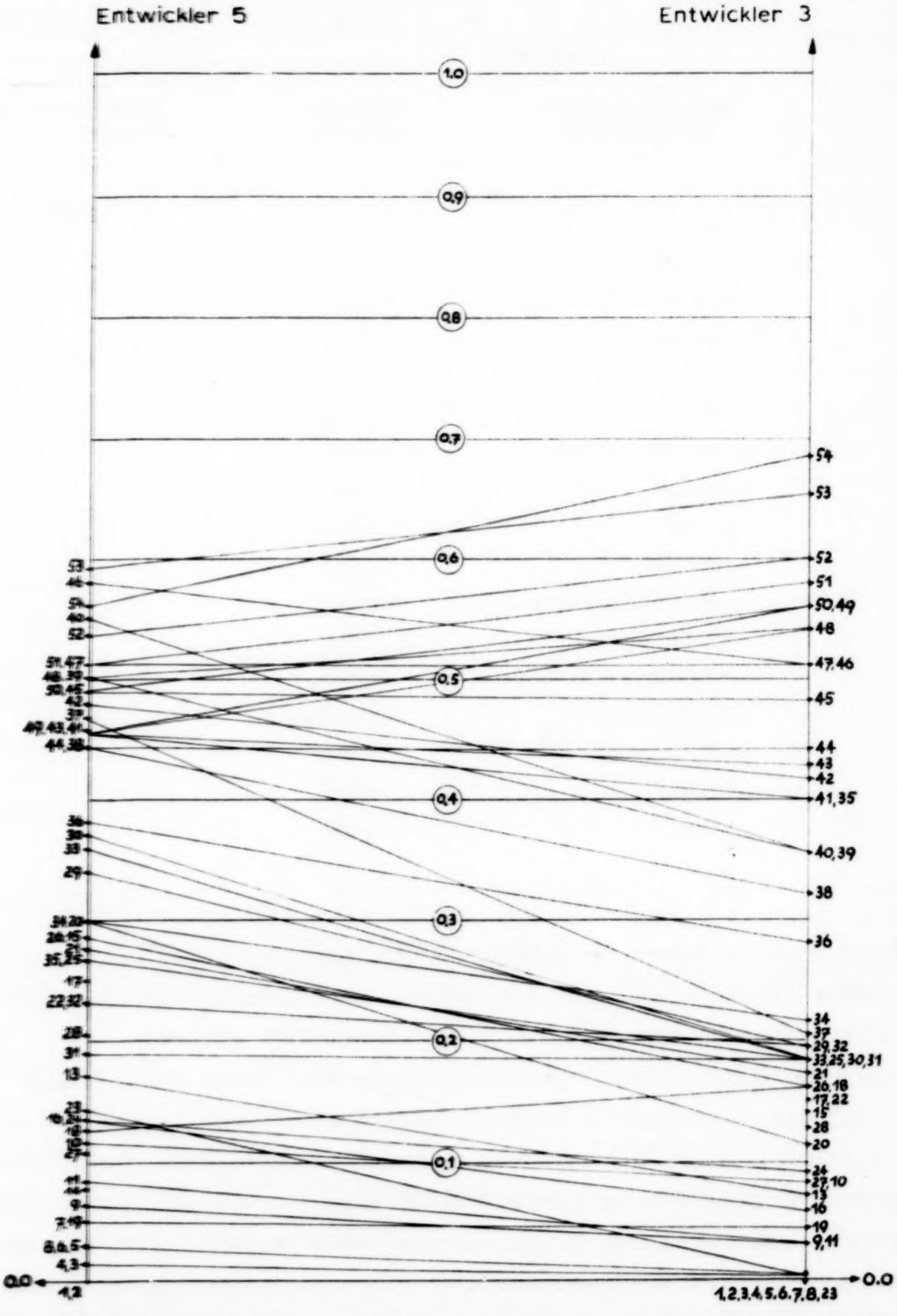


Fig. 4. Kieselgel G-Platten mit: E-3 = Cyclohexan–Chloroform–Diäthylamin (5:4:1) und E-5 = Benzol–Äthylacetat–Diäthylamin (7:2:1). Die den Zahlen entsprechenden Alkaloide sind aus Tabelle I ersichtlich.

Auf die laufenden Startpunkte einer Kieselgelplatte werden daher 1 × 5 mm³ bis 10 × 5 mm³ der Analysenlösung aufgetropft und zusätzlich 1 × 5 mm³ der Rhodamin B-Lösung als Vergleich auf einen Startpunkt aufgebracht. Die Platte wird in Entwickler 3 eingestellt und nach den Angaben im experimentellen Teil sichtbar gemacht.

Alkaloide mit R_F-Werten über 0.3

Diese Alkaloide gehören zur Gruppe II. Aus dem auf Rhodamin B (R_F-Wert = 0.20) korrigierten R_F-Wert und auf Grund der Anfärbung mit Jodplateat-Reagenz hat man bereits Anhaltspunkte für die Identität des gesuchten Alkaloids. Die weitere Identifizierung erfolgt auf Kieselgelplatten mit der günstigsten Auftragsmenge (um 50 μg) in den Entwicklern 4 und 5.

Die mutmasslichen Alkaloide trägt man ebenfalls auf einzelne Startpunkte getrennt und auch zusammen mit der Analysenlösung auf. Nach der Entwicklung kann man einzelne Felder mit Glasplatten der Grösse 20 × 10 cm abdecken. Diese können dann mit weiteren Sprühreagenzien, die auf spezifische Gruppen reagieren, besprüht werden.

Alkaloide, die in Entwickler 3 unterhalb R_F-Werten von 0.3 liegen

Diese gehören zur Gruppe I. Zunächst wird eine Kieselgelplatte mit günstigen Auftragsmengen in E-2 entwickelt und die R_F-Werte (auf Rhodamin B = R_F 0.49) bestimmt. Auch hier deckt man einige Felder mit Glasplatten ab, um später spezifische Anfärbereaktionen durchführen zu können. Schliesslich werden zwei weitere Kieselgelplatten mit den mutmasslichen Alkaloiden allein und mit Analysenlösung auf den Startpunkten in die Entwickler 1 und 2 gebracht. In Zweifelsfällen können auch basische Platten nach Fig. 2 (a–c) zu Rate gezogen werden.

Mit reinen Alkaloidlösungen ist die Analyse ohne weiteres durchführbar. Bei der praktischen Analyse wird man damit rechnen müssen, dass bereits Zersetzungsprodukte oder auch andere mit Jodplateat reagierende Stoffe in der Analysenlösung vorhanden sind, die Schwierigkeiten bereiten. Wegen der Begleitstoffe, die die R_F-Werte verändern können (Verdrängung) wird es nützlich sein, den einen oder den anderen Startpunkt mit einem Alkaloid an der Grenze der Gruppen, z.B. Nr. 36 oder Nr. 38, zu versehen. Schliesslich ist zu bemerken, dass Tabelle I noch durch die fehlenden Alkaloide ergänzt werden kann. In gleicher Weise wie Alkaloide lassen sich auch andere Stoffgruppen auf Platten systematisch darstellen.

EXPERIMENTELLER TEIL

Zur Ausführung unserer Versuche diente die Grundausrüstung nach STAHL*. Die Schichten auf 20 × 20 cm Glasplatten wurden nach Angaben den der Grundausrüstung beigefügten Vorschriften gestrichen und anschliessend aktiviert.

Die uns zur Verfügung stehenden reinen Alkaloide lösten wir 1 %ig in Äthylalkohol. Mit einer Glaskapillare, ⌀ 1 mm, wurden jeweils 2 × 2.5 mm^3 Lösung, entsprechend 50 μg Substanz, auf die Startpunkte aufgebracht. Auf einem oder mehreren Startpunkten wurde jeweils zusätzlich der in Tabelle I angegebene Vergleichsfarbstoff mitaufgetragen. Es handelte sich dabei um eine 0.5 %ige Lösung von Rhodamin B oder Dimethylaminoazobenzol (Buttergelb). Die Startpunkte waren 15 mm vom unteren Plattenrand entfernt, und die Frontlinie wurde in 100 mm Abstand von den Startpunkten mit Bleistift eingraviert (siehe Fig. 1).

Der zur Entwicklung benötigte Tank wurde mit 100 ml Lösungsmittelgemisch gefüllt. Als Lösungsmittel verwendeten wir die Sorten „für Chromatographie" Merck.

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* Zu beziehen bei Fa. Desaga GmbH, Heidelberg, Hauptstr. 60.

Auf die genaue Zusammensetzung des Entwicklers war ganz besonders zu achten. Jeder angegebene Volumenteil der Lösungsmittel wurde für sich abgemessen. So wurde der Diäthylaminanteil jeweils abpipettiert, und die anderen Lösungsmittelpartner jeder für sich im Messzylinder abgemessen. Die Entwickler mussten nach 3–4-fachem Gebrauch täglich erneuert werden, da der flüchtige Anteil abdampfte. Vor dem Einstellen der Platten schüttelten wir das Gemisch bei aufgesetztem Schliffdeckel kräftig durch; auch das zusätzliche Einlegen eines Papierstreifens erwies sich für eine gleichmässige Lösungsmittelfront als günstig. Nach Einstellen der Dünnschichtplatten in das Gefäss stieg der Entwickler innerhalb von 20–40 Minuten bis zur eingravierten Höhe. Nach Entnahme der Platten verdunsteten die Eluentien ziemlich rasch an der Luft, so dass sie gleich mit filtriertem U.V.-Licht betrachtet und dann angesprüht werden konnten.

Als Anfärbereagenz verwendeten wir modifiziertes Jodplateat-Reagenz, bestehend aus einem Gemisch gleicher Volumenteile einer 0.3%igen wässrigen Platinchloridchlorwasserstofflösung und einer 6%igen wässrigen Kaliumjodidlösung. Es wurde intensiv gesprüht, so dass die Plattenschicht ganz durchfeuchtet wurde (Tropfenbildung vermeiden).

Das Sprühreagenz gab für die meisten Alkaloide eine genügende Differenzierung, da teilweise nicht nur Farbunterschiede, sondern auch Farbänderungen während und nach dem Besprühen wahrgenommen werden konnten. Einige wenige Alkaloide traten erst nach einigen Minuten in Erscheinung, wenn das Reagenz abtrocknete. Die Färbungen der Alkaloide mit Jodplateatreagenz waren auch von der Konzentration des aufgebrachten Alkaloids abhängig. So war der Rand oft anders gefärbt als der Kern. Die in Tabelle I angegebenen Anfärbungen mit Jodplateatreagenz sind also nur bedingt richtig und durch entsprechende Vergleiche zu bestätigen. Andere spezifische Anfärbereagenzien sind in der Literatur beschrieben[14–19].

ZUSAMMENFASSUNG

Alkaloide lassen sich am schnellsten adsorptionschromatographisch auf Dünnschichtplatten entwickeln. Das Verhalten von über 50 Alkaloiden in verschiedenen Entwicklern ermöglicht es, zwei Gruppen von Alkaloiden zu bilden. Nach Ermittlung der Gruppenzugehörigkeit können die Alkaloide auf Grund der in Tabellen angegebenen R_F-Werte und Anfärbbarkeit identifiziert werden.

SUMMARY

The most rapid method of separating alkaloids is thin-layer chromatography. From investigations with more than 50 alkaloids it was found that the alkaloids can be divided into two groups, according to their behaviour with various solvent systems. After establishing to which group an alkaloid belongs, it can be identified by means of the table of R_F values and colour reactions, given in this paper.

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GAS-LIQUID CHROMATOGRAPHY OF LINEAR DETERGENT ALKYLATES

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(Received October 29th, 1960)

Although a multitude of anionic surfactant types have been synthesized and tested during the past two decades, one of the types to have achieved major commercial importance is the alkyl-aryl sulphonate type.

In considering these compounds it is evident that it is the hydrophobic part of the molecule which governs the efficiency of the surfactant. Information about this hydrophobic, or hydrocarbon, part of the molecule is then of major importance, and owing to the difficulty of examination by conventional chemical techniques this study was undertaken.

Alkylates supplied commercially can be divided into two main groups, those formed by alkylation of benzene with tetrapropylene, and those formed by alkylation of benzene with a straight chain olefin. Owing to the extremely complex nature of the tetrapropylene benzene types, satisfactory separations have not so far been obtained. Alkylates prepared from *n*-olefins (linear alkylates), however, represent much more simple systems. This is evident from the fact that linear types have a very restricted number of isomeric variations for each possible aliphatic chain length. Table I readily illustrates this limitation.

TABLE I

NUMBER OF POSITIONAL ISOMERS OF MONOPHENYL-*n*-ALKANES

Aliphatic chain	*Number of carbon atoms*	*Number of possible phenyl positions*
Hexane	6	3
Heptane	7	4
Octane	8	4
Nonane	9	5
Decane	10	5
Dodecane	12	6

As can be seen from this table, the number of possible isomeric phenyl positions can be calculated as being (Number of C-atoms/2) where fractional values are rounded upward to the nearest whole number. Current commercial linear alkylates, Shell's Dob 83 and Dobane JN being examples, generally contain significant amounts of aliphatic chain lengths other than dodecyl.

J. Chromatog., 6 (1961) 74–79

This paper confirms this and gives a characterisation of chain lengths together with the spread of phenyl attachment on these chains.

EXPERIMENTAL

Apparatus

A Pye argon chromatograph* with argon as carrier gas.

Columns

All columns were of glass 4 ft. long and ¼ in. I.D. The stationary phase which effected best separation was Apiezon L grease. This was dissolved in a volatile solvent, 40–60° light petroleum, slurried with Celite (Embacel by May and Baker, 60–100 mesh), oven dried, and packed by vibration.

Operating conditions

Separations were carried out at 130°, using a detector voltage of 1750 V, a sensitivity of "× 10" and a flow rate of 40 ml per min of Argon (measured by a soap bubble manometer). Inlet pressure was 10 lb./sq.in. and sample size was 0.05 μl. Under these conditions the column efficiency was approximately 650 theoretical plates per foot.

DISCUSSION

Figs. 1 and 2 show the chromatograms obtained under standard conditions for Dob 83 and Dobane JN. The problem of identifying these peaks was overcome in several stages. It was observed that the peaks occurred in definite groups and it seemed

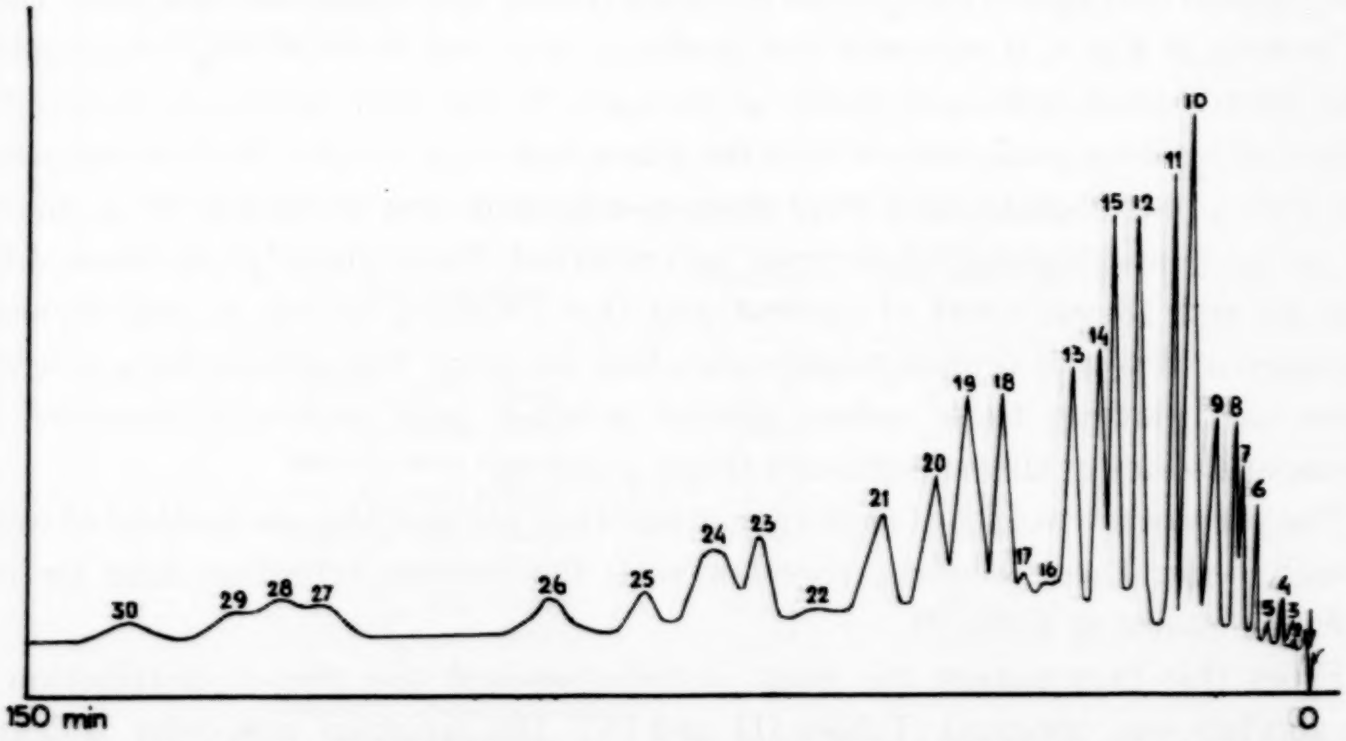

Fig. 1. Chromatogram of Dob 83. Sample size: 0.05 μl; column: 5 % w/w Apiezon L/(60–100 mesh) Celite (4 ft. long); temp.: 130°; flow rate: 40 ml argon/min; inlet pressure: 10 lb./sq. in.; outlet pressure: atmospheric; detector voltage: 1750 V; sensitivity: × 10; chart speed: 6 in./h. For key to peak numbers see Table II.

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* W. G. Pye and Co. Ltd., Granta Works, Cambridge.

likely that, in general, these would be of similar molecular weight, at a first approximation. Calculation of retention volumes showed that some of the sets of peaks were common to both materials but, particularly in Dob 83, there were extra sets at lower molecular weights.

A personal communication from DENZLER[1] had indicated that there was no sign, from mass spectrometric evidence, of primary alkylbenzenes, thus reducing the number of possible isomers, given in Table I of the introduction, by one in each case. It then seemed logical that isomerisation had occurred during alkylation. At first sight therefore, looking at Fig. 1, it was seen that peaks 17 to 21 and 22 to 26 might be regarded as the phenyl-undecanes and phenyl-dodecanes. It was then necessary to ascertain in which order these peaks were within the given molecular weight. Marker compounds 1-, 2- and 3-phenyl-dodecanes were then synthesised and a sample of a mixture of 2-, 3-, 4-, 5- and 6-phenyl-dodecanes was received. These immediately showed that peaks 23 and 18 were out of context and that twinning of the 5- and 6-phenylundecanes, and 5- and 6-phenyl-dodecanes had occurred. Calculating $\log_{10}$ retention volume and plotting these values against selected peak numbers[2] identified the homologous series of the substituents (Figs. 3 and 4).

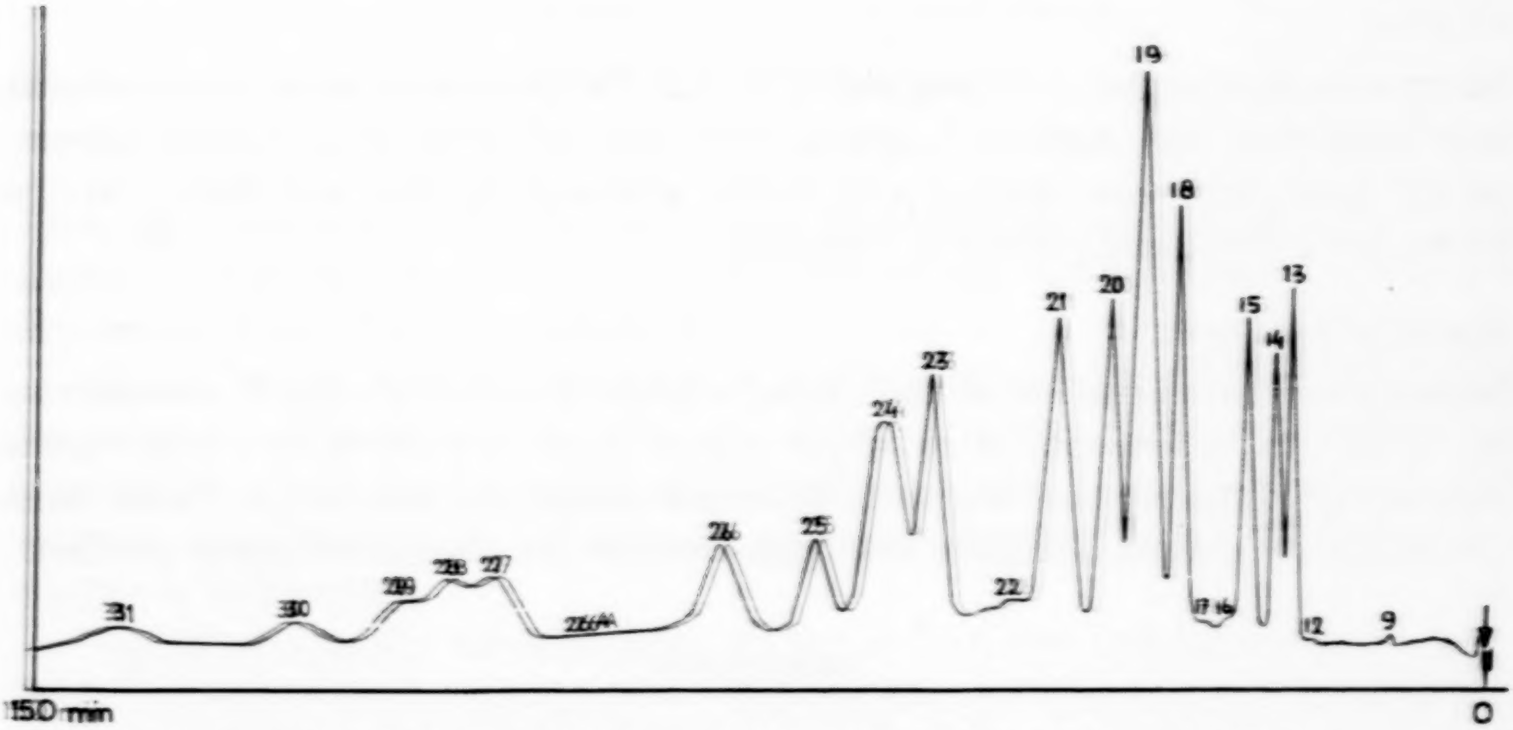

Fig. 2. Chromatogram of Dobane JN. For conditions see legend to Fig. 1.

The percentage weight of each isomer was then obtained by the method of weighing each peak. These weights, together with the relative retention data for each isomer are shown in Table III.

From this information the mass distribution and the phenyl distribution for each alkylate was obtained (Tables III and IV). The apparent molecular weight of each alkylate calculated from these data compared favourably with the molecular weight obtained by ebulliometry.

In addition to the peaks which were positively identified there were four remaining unidentified peaks. These peaks, 16, 17, 22 and 26A account for 6.52 % of the total

TABLE II

EXAMINATION OF SHELL ALKYLATES DOB 83 AND DOBANE JN
(Retention volumes relative to *n*-hexadecane = 1.00)

Peak No.	*Isomer*	*Dob 83* Relative retention volume	*Dob 83* Area of each peak, % of total area	*Dobane JN* Relative retention volume	*Dobane JN* Area of each peak, % of total area
1	3-Phenylpentane	0.04	0.03		
2	2-Phenylpentane	0.05	0.14		
3	3-Phenylhexane	0.07	0.06		
4	2-Phenylhexane	0.09	0.25		
5	4- and 3-Phenylheptane	0.14	0.25		
6	2-Phenylheptane	0.17	0.86		
7	4-Phenyloctane	0.21	1.19		
8	3-Phenyloctane	0.22	1.63		
9	2-Phenyloctane	0.28	2.90	0.28	0.05
10	5- and 4-Phenylnonane	0.35	6.49		
11	3-Phenylnonane	0.40	5.33		
12	2-Phenylnonane	0.52	6.08	0.52	0.18
13	5-Phenyldecane	0.58	5.97	0.58	3.73
14	4-Phenyldecane	0.63	4.94	0.63	3.47
15	3-Phenyldecane	0.71	6.10	0.71	4.76
18	2-Phenyldecane	0.92	5.75	0.92	7.27
19	6- and 5-Phenylundecane	1.03	8.15	1.03	13.33
20	4-Phenylundecane	1.11	5.41	1.11	7.97
21	3-Phenylundecane	1.27	6.30	1.27	9.06
23	2-Phenylundecane	1.66	4.28	1.66	8.72
24	6- and 5-Phenyldodecane	1.80	5.94	1.80	11.02
25	4-Phenyldodecane	2.00	2.54	2.00	4.76
26	3-Phenyldodecane	2.28	3.37	2.28	6.73
27	2-Phenyldodecane	2.96	2.27	2.96	4.04
28	7-Phenyltridecane	3.08	2.04	3.08	3.60
29	6- and 5-Phenyltridecane	3.25	1.80	3.25	2.87
30	4-Phenyltridecane	3.54	1.19	3.54	1.29
31	3-Phenyltridecane	4.05	1.24	4.05	2.64
32	2-Phenyltridecane	5.31	1.10	5.31	1.04

TABLE III

MASS DISTRIBUTION OF SHELL ALKYLATES DOB 83 AND DOBANE JN

Mass distribution	*Shell Dob 83 (% wt.)*	*Shell Dobane JN (% wt.)*
148 (C_5 side chain)	0.17	—
162 (C_6 side chain)	0.31	—
176 (C_7 side chain)	1.01	—
190 (C_8 side chain)	5.72	0.05
204 (C_9 side chain)	17.90	0.18
218 (C_{10} side chain)	22.76	19.23
232 (C_{11} side chain)	24.14	38.18
246 (C_{12} side chain)	14.12	26.55
260 (C_{13} side chain)	7.37	11.44
Assorted branched side chain alkylates	6.52	4.38
Average mol. weight*	225.0	238.2
M.W. by ebulliometry	226.0	238.0

* Assuming average molecular weight for assorted branched side chain alkylates.

TABLE IV

WEIGHT DISTRIBUTION BY PHENYL POSITION OF SHELL ALKYLATES DOB 83 AND DOBANE JN

Phenyl position	*Shell Dob 83 (% wt.)*	*Shell Dobane JN (% wt.)*
1-Phenyl	0.0	0.0
2-Phenyl	23.63	21.25
3-Phenyl	24.19	23.19
4-Phenyl	18.90	16.59
5-Phenyl	17.17	17.34
6-Phenyl	7.95	13.61
7-Phenyl	2.04	3.60
Branched side chain	6.52	4.38

weight in Dob 83, and 4.38% of the total weight in Dobane JN. These unidentified peaks are probably due to branched side chain alkylates.

Small amounts of C_{14} alkyl chains are also known to be present in both alkylates but the peaks were too small and diffuse to be examined.

No peaks were found for the 1-phenyl-*n*-alkanes thus confirming the mass spectrometric evidence.

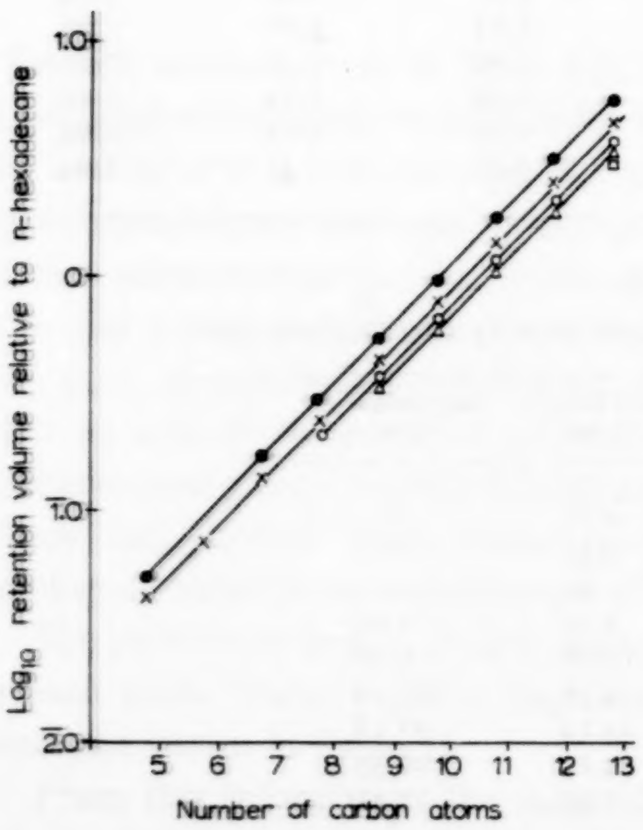

Fig. 3. Relationship between $\log_{10}$ retention volume and number of carbon atoms for Dob 83. ● = 2-phenyl isomers; × = 3-phenyl isomers; ○ = 4-phenyl isomers; △ = 5-phenyl and 5- and 6-phenyl isomers; □ = 7-phenyl isomers.

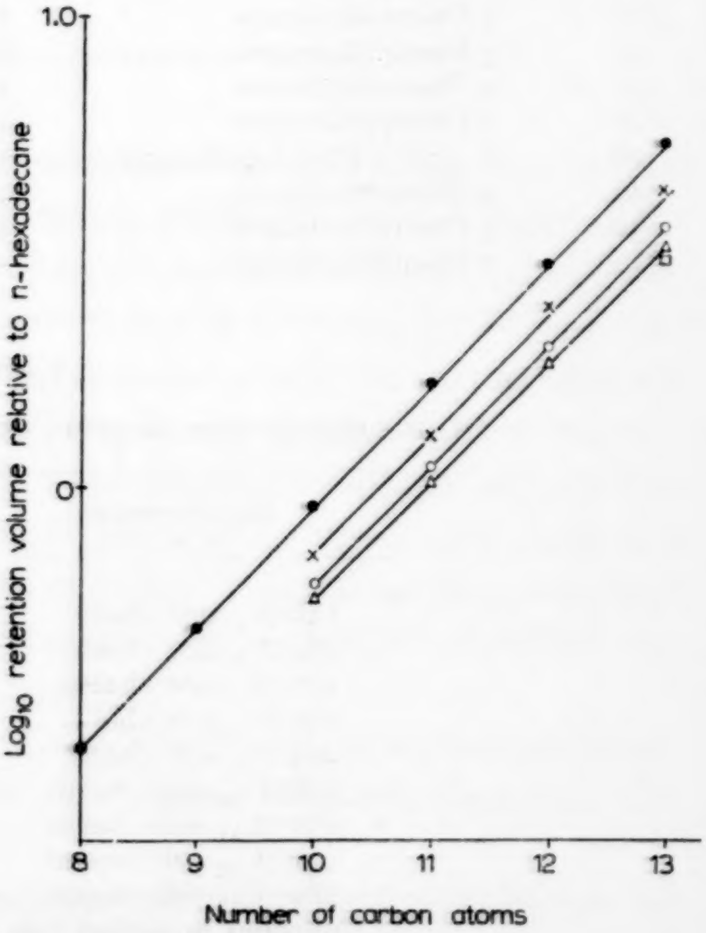

Fig. 4. Relationship between $\log_{10}$ retention volume and number of carbon atoms for Dobane JN. ● = 2-phenyl isomers; × = 3-phenyl isomers; ○ = 4-phenyl isomers; △ = 5- and 6-phenyl isomers; □ = 7-phenyl isomers.

ACKNOWLEDGEMENT

We wish to express our thanks to Mr. I. D. BURGESS (Colgate-Palmolive Ltd.) for synthesizing marker compounds, including 1-, 2- and 3-phenyl-dodecane, and to Dr. A. MCLEAN (British Hydrocarbon Chemicals Limited, Grangemouth) for supplying samples of 2-phenyl-dodecane and a mixture of 2-, 3-, 4-, 5- and 6-phenyl-dodecanes. We are also indebted to Colgate-Palmolive for granting permission to publish this paper.

SUMMARY

Apiezon L grease was found to be a selective liquid substrate for separating linear detergent alkylates by gas-liquid chromatography. The most important point which arises from this work is that substantially complete identification has been achieved of the components of a commercial alkylbenzene. Retention volumes, relative to *n*-hexadecane, are given for all the compounds appearing in two commercial samples of linear detergent alkylate, namely Shell's Dob 83, and Dobane JN.

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IMPROVED GLASS-PLATE HEATER FOR PAPER CHROMATOGRAPHY

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(Received October 31st, 1960)

Heating usually is the final step necessary for detecting carbohydrates and related substances that have been separated on paper by a developing solvent and treated with a suitable reagent for producing colored spots. The heating step may be as critical as the two preceding for producing an informative chromatogram, and requires more precise control than often is achieved. The advisability of controlled temperature and length of heating seems to be implied because these conditions are always included in any complete description of each detecting reagent.

Only one[1] of a number of excellent books and reviews covering paper chromatography of carbohydrates discusses devices for heating chromatograms to develop colored spots. Among devices recommended are the infrared lamp and radiating electric space heater[1], hot plate[2], and "oven"[2]. ALBON AND GROSS[3] described a specially designed oven, with glass doors, that controls temperature precisely and uniformly. The glass-plate heater of FETZER AND OUGH[4] also offers the advantage of visibility of the paper during heating as well as simplicity and compactness of construction. Since we found no commercially available device which offered any of these features, we modified the FETZER–OUGH heater to improve control and uniformity of temperature as well as to provide greater convenience and facility for handling damp sheets of fragile paper.

DETAILS OF CONSTRUCTION AND USE

The improved heater as constructed at the Northern Regional Laboratory is pictured in Fig. 1. The box, which has an overall dimension of 10½ × 20 × 26 in. is made of ½-in. asbestos board, reinforced at the corners with galvanized sheet steel, and is lined with aluminum foil. The bottom is insulated with a 2-in. sheet of fiber glass. At the top stainless-steel rails support the full-width window of heat-resistant glass and form the channel in which the stainless-steel frame for chromatograms slides. A door 7-in. high forms the front side of the box and drops down horizontally to permit maintenance. Above the door is an open slit ¼-in. wide for inserting the stainless-steel frame on which the paper chromatogram is supported.

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* This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

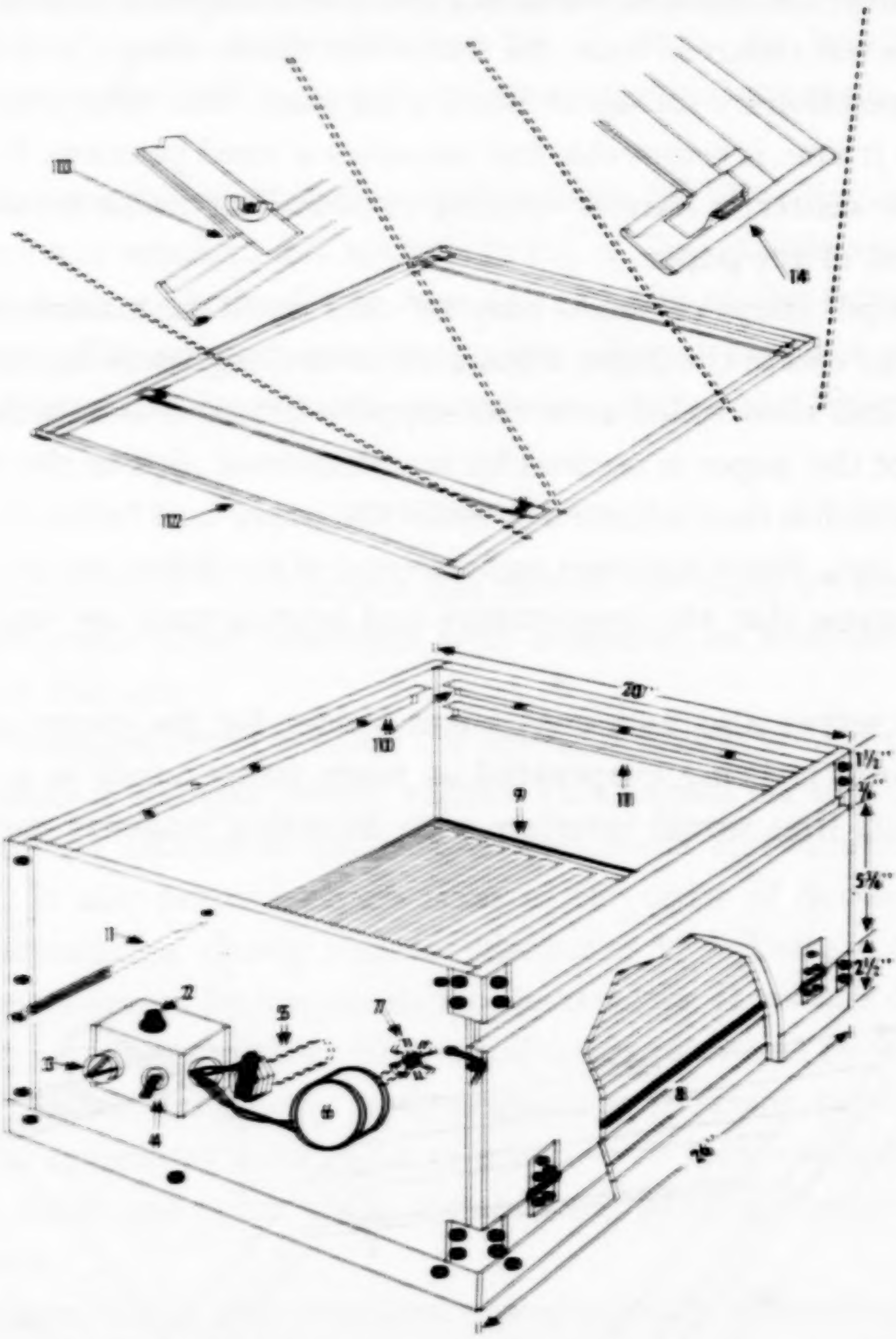

Fig. 1. Diagram of heater and frame for holding paper chromatograms. 1 = Thermometer. 2 = Fuse. 3 = Pilot light. 4 = Switch. 5 = Thermoswitch. 6 = Fan motor. 7 = Fan. 8 = Fiber glass insulation. 9 = Heater plate. 10 = Support for glass top. 11 = Track for frame. 12 = Frame for paper sheets or strips. 13 = Detail of adjustable support. 14 = Detail of stationary support.

The heating element is a Ra-Grid heater-plate*, Model K, 24 × 12 × 7/32 in., for 115 V and 950 W. It rests on ½-in. fiber glass blocks and is controlled by a Fenwal Thermoswitch located about 3 in. above the plate. Any desired temperature up to 150° is readily obtained and maintained; the warm-up time is 15–20 min. A thermometer projects about 7 in. into the box at the left of the regulator and a small radio-tube cooling electric fan (2½-in.-blade diameter) operated through a constant duty,

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* A product of the Blue Ridge Glass Corporation, Kingsport, Tennessee. Trade names are given as part of the exact specifications, not as a recommendation or an endorsement of the products named over those of other manufacturers.

1/20 h.p. motor, projects into the box at the right of the regulator. When running, the fan reduces temperature lag in the box to about ± 2°.

The adjustable, stainless-steel frame (item 12 of Fig. 1) has overall dimensions of 62.8 × 48 cm. It is made of strips of light-weight stainless steel, with edges rolled to increase rigidity; the finished width is 2 cm. The crosspiece, shown at the left in the frame, usually is not removed from the frame but slides along the sides and is secured at any desired position by means of small wing nuts. The other crosspiece, shown at the right in the frame, is removable but occupies a fixed position. It is folded lengthwise through the center; a narrow opening extends lengthwise between the edges for inserting one end of the paper.

Any size paper strips or sheets may be used up to the maximum Whatman 46 × 57-cm sheets. One end of the paper is inserted in the lengthwise opening in the stationary crosspiece and then rolled over the support before it is attached to the frame. The other end of the paper is secured by stainless-steel clips to the movable support, the position of which is then adjusted to make the paper taut before inserting the frame into the heated box. Since insertion and removal of the frame are accomplished quickly through a narrow slot, the temperature and heating time are uniform for all parts of the sheet.

The frame serves also as a convenient holder for the chromatogram while the developing solvent is being evaporated at room temperature in a fume hood. Last traces of solvents that would interfere with detecting reagents may be removed by placing the paper-filled frame briefly in the heater already slightly warmed. The paper can also be kept in the frame while the detecting reagent is being applied, as well as during washing and drying after color development.

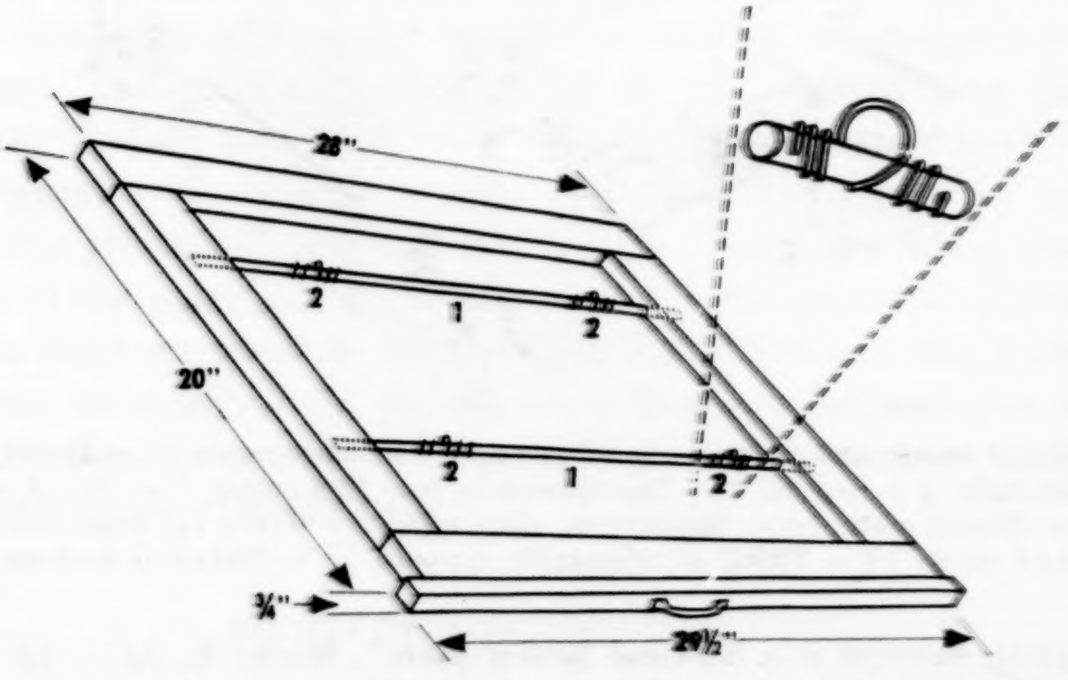

Fig. 2. Diagram of frame for heater fabricated with wood. 1 = Stainless-steel rod, ¼-in. diameter 2 = Sliding fasteners.

When desired, humidity in the box can be increased by placing a narrow container of hot water between the heater plate and the front of the box.

Fumes from heating chromatograms may be removed by adaptation of a small vent and exhaust fan at the back of the box.

Alternatively, as constructed at the Clinton Corn Processing Company, the cabinet may be fabricated with wood from commercial 1-in. material; other features remained essentially the same, except the insulating liner of aluminum foil was omitted. In this case inside dimensions are 20 × 28 in. for breadth and length, respectively. The frame for holding sprayed chromatograms was made from $^3/_4 \times 2$ in. wood strips (Fig. 2).

A diagram showing the construction and facilities for attaching sprayed chromatograms, 26 × 57 cm or smaller, also appears in Fig. 2. Two stainless-steels rods ($^1/_4$ in. diameter) are set flush with the lower face at the sides of the frame and centered about 27 cm apart. The sprayed chromatogram, clipped to glass rods, is held by sliding fasteners (Fig. 2) fashioned with copper wire (Brown and Sharpe gage No. 14).

DISCUSSION

This simple and easily constructed heater has met all our needs for bringing out color spots on chromatograms after treatment with reagents. In addition, it has several very advantageous features.

The main advantages result because the operator is able to heat the entire paper sheet uniformly while it remains clearly visible. This feature permits accurate control of heating time so that maximum contrast between spots and minimum background color is achieved. It also permits observation of the order of development of color which aids in differentiating closely related substances,[2,5–8]. Transient colors may be observed before replacement by less distinctive final colors. It enables optimum length of heating to bring out the sensitive, differential colors produced by some of the most useful reagents[9–11] which aid greatly in distinguishing different types of sugars. Our observations are in agreement with those of others[7,10,12] that overheating obliterates the differences in shade and color which are obtained from numerous reagents under optimum conditions.

Other advantages derive from excellent reproducibility of results and from ability to make valid comparisons on large sheets of paper for identification and for estimation or measurement of relative quantities.

Any temperature within the wide range necessary for carbohydrates can be obtained readily. Adaptation may be made for special requirements, such as increasing the humidity in the heating chamber[13]. Loss of time and of valuable materials through accident is precluded by the sureness and facility with which the fragile paper sheets are handled on the adjustable frame.

ACKNOWLEDGEMENT

Acknowledgement is made to CHRISTIAN SCHWABE, presently at the College of Dentistry, State University of Iowa, Iowa City, for the important suggestions he contributed relative to design while at Clinton.

SUMMARY

An easily made heater is described for bringing out colored spots on paper chromatograms after the detecting reagent has been applied. The device permits precise control of length and temperature of heating as well as heating the entire paper uniformly while it is clearly visible to the operator. The paper is supported on a versatile frame. Paper sheets or strips of different sizes can be accommodated.

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Short Communications

Chromatographie sur papier d'éléments transuraniens

Variation des R_F du plutonium et de l'américium dans les mélanges *n*-butanol–HCl (1:1)

Jusqu'à présent, la chromatographie sur papier des éléments transuraniens a fait l'objet de peu de travaux. Dès 1949, FINK ET FINK[1] donnaient, cependant, les R_F de Pu(III), Pu(IV) et Pu(VI) dans différents solvants et, en particulier, dans des mélanges à base de méthyléthylcétone. Plus récemment, ENGLISH ET FOREMAN[2] ont indiqué les séparations suivantes par chromatographie sur papier: Pu(IV)–Pu(VI) par butanol–HCl 3 *M*; Pu(III)–Pu(IV) par éthylacétate–HCl concentré, et une séparation du Pu(III) des actinides de numéro atomique plus élevé par éthylacétate–HNO_3–$NaNO_2$.

Cette note indique la variation des valeurs R_F à 20° de U(IV); U(VI); Pu(III); Pu(IV); Pu(VI) et Am(III) en fonction de la concentration en HCl de mélanges *n*-butanol–HCl (1:1). Pour ces mélanges, la détermination des R_F de la plupart des métaux jusqu'à l'uranium a été faite par GUEDES DE CARVALHO[3].

Obtention des différents états de valence des éléments étudiés

(1) *Solution d'uranium (IV)*. Réduction d'un sel d'uranyl par Sn^{2+} dans HCl concentré. Lorsqu'on estime la réduction suffisante, on précipite UF_4 vert par HF. Après lavage, le précipité est redissous par HCl concentré en présence de BO_3H_3. On précipite ensuite $U(OH)_4$ par le gaz ammoniac. Après lavage le précipité d'hydroxyde est redissous par HCl 1 *M*.

(2) *Solution d'uranium (VI)*. Solution de chlorure d'uranyl.

(3) *Solution de Pu (III)*. Obtenue par réduction d'une solution HCl 0.5 *M* de ^{239}Pu par le chlorhydrate d'hydrazine. A 4 ml de solution de ^{239}Pu (env. 1 mg), on ajoute 1 ml d'une solution 0.2 *M* de N_2H_5Cl; on maintient au moins une heure à 95–100°. On obtient une solution bleu-violet donnant une bande d'absorption caractéristique à 470 mμ[4].

(4) *Solution de Pu (IV)*. Solution nitrique obtenue par oxydation spontanée du Pu(III) en Pu(IV) en milieu HNO_3 concentré.

Une solution HCl 0.5 *M* de Pu(III), préparée comme précédemment est évaporée au bain-marie. Le résidu est repris par HNO_3 concentré (d = 1.33, environ 11 *M*). La solution devient vert brillant par suite de la formation du complexe nitraté de Pu(IV). Cette solution nous a donné le spectre d'absorption caractéristique de Pu(IV) en milieu nitrique concentré[4].

(5) *Solution de Pu(VI)*. Solution nitrique obtenue selon la méthode de Kraus: oxydation de Pu(IV) en solution HNO_3 0.5 *M* chauffée 24 h à 80° ± 5°[4].

(6) *Solution d'Am(III)*. Solution HCl 1 *M* de ^{241}Am.

Réalisation des chromatogrammes

Nous avons utilisé la technique ascendante: feuilles de papier Whatman No. 1 de 420 × 280 mm roulées et posées sur le fond de cuves cylindriques recouvert de solvant.

Les molarités HCl des mélanges *n*-butanol–HCl (1:1) étudiés sont: 1 *M*; 2 *M*; 3 *M*; 4 *M*; 5 *M*; 6 *M*; 8 *M*; et 10 *M*. Jusqu'à 4 *M* on a deux phases liquides; dans ce cas, une partie de la phase aqueuse placée dans un petit bécher est introduite dans la cuve dont l'atmosphère est ainsi saturée par la phase aqueuse et la phase organique. Sur les feuilles de papier on dépose six spots: Pu(III), Pu(IV), Pu(VI), Am(III), U(IV), U(VI), que l'on sèche à l'air chaud.

Le développement des chromatogrammes s'effectue à 20° ± 0.5°, pendant environ 20 heures, dans une grande étuve spécialement étudiée[5] où on a placé les cuves.

Analyse des chromatogrammes

(1) *Révélation chimique. Mise en évidence d'une réaction spécifique du Pu(VI)*. Après séchage, les chromatogrammes sont vaporisés avec une solution ammoniacale de 8-hydroxyquinoline. On observe:

(a) sur les pistes de Pu(III) et de Pu(IV), respectivement une seule tache jaune orangé;

(b) sur les pistes U(IV) et U(VI), respectivement une seule tache orangée;

(c) sur les pistes de Am(III), rien, cet élément se trouvait en quantité beaucoup plus faible;

(d) sur les pistes Pu(VI), deux taches, une jaune orangé et une noire de R_F plus élevé, cette dernière n'étant pratiquement plus visible pour les molarités HCl supérieures à 4 *M*.

Toutes ces taches apparaissent sombres en lumière ultra-violette.

La tache noire observée sur les pistes de Pu(VI) semble caractéristique de cet état de valence du Pu, puisque Pu(III) et Pu(IV) donnent des taches identiques jaune orangé. Ces colorations sont indépendantes des anions présents dans la solution de Pu. Nous l'avons vérifié en déposant sur une bande de papier Whatman No. 1, un spot de Pu(III) en solution HCl, un spot de Pu(IV) en solution HNO_3, un spot de Pu(IV) en solution HCl, un spot de Pu(VI) en solution HNO_3, un spot de Pu(VI) en solution HCl. On vaporise de 8-hydroxyquinoline ammoniacale: tous les spots de Pu(III) et Pu(IV) donnent une coloration jaune orangé alors que ceux de Pu(VI) se colorent en noir.

Un essai de sensibilité de ces réactions colorées nous a donné: 0.2 μg pour Pu(III) et Pu(IV) et 0.08 μg pour Pu(VI).

(2) *Examen des chromatogrammes par autoradiographie*. Les chromatogrammes ont ensuite été mis en contact, en chambre noire, avec des films R. X. Kodak Standard, pendant une dizaine de jours. Les films sont ensuite développés. Voir Fig. 1.

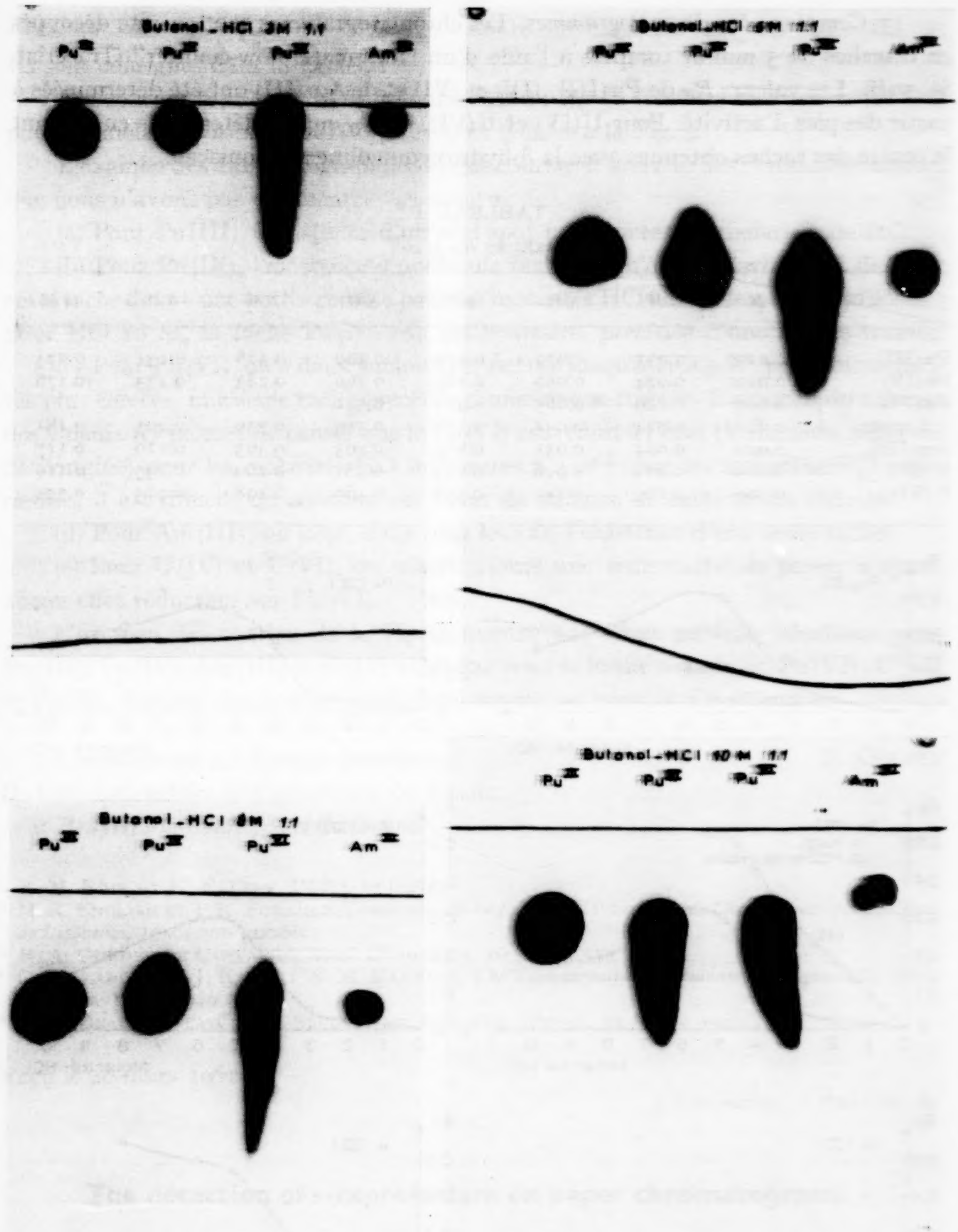
Butanol - HCl 3M 1:1
Pu III
Pu IV
Pu VI
Am III
Butanol-HCl 6M 1:1
Pu III
Pu IV
Pu VI
Am III
Butanol - HCl 8M 1:1
Pu III
Pu IV
Pu VI
Am III
Butanol - HCl 10M 1:1
Pu III
Pu IV
Pu VI
Am III

Fig. 1. Autoradiogrammes de Pu (III), Pu (IV), Pu (VI) et Am (III) dans des mélanges *n*-butanol–HCl (1:1).

(3) *Comptage des chromatogrammes.* Les chromatogrammes sont ensuite découpés en tranches de 5 mm et comptés à l'aide d'un "automatic flow-counter" (Tracerlab SC 50B). Les valeurs R_F de Pu (III), (IV) et (VI) et de Am (III) ont été déterminées à partir des pics d'activité. Pour U (IV) et U (VI), les R_F ont été déterminés en prenant le centre des taches obtenues avec la 8-hydroxyquinoline ammoniacale.

TABLEAU I

VALEURS R_F À 20°

HCl	*1 M*	*2 M*	*3 M*	*4 M*	*5 M*	*6 M*	*8 M*	*10 M*
Pu (III)	0.009	0.035	0.075	0.179	0.309	0.316	0.231	0.174
Pu (IV)	0.020	0.024	0.050	0.180	0.260	0.283	0.233	0.176
Pu (VI)	0.099	0.189	0.285	0.421	0.541			
2 spots	0.020	0.024	0.051	0.158	0.309	0.330	0.205	0.181
Am (III)	0.020	0.024	0.051	0.160	0.293	0.303	0.219	0.117
U (IV)	≃ 0.000	0.014	0.026	0.133	0.281	0.269	0.195	0.110
U (VI)	0.106	0.169	0.241	0.358	0.469	0.498	0.475	0.555

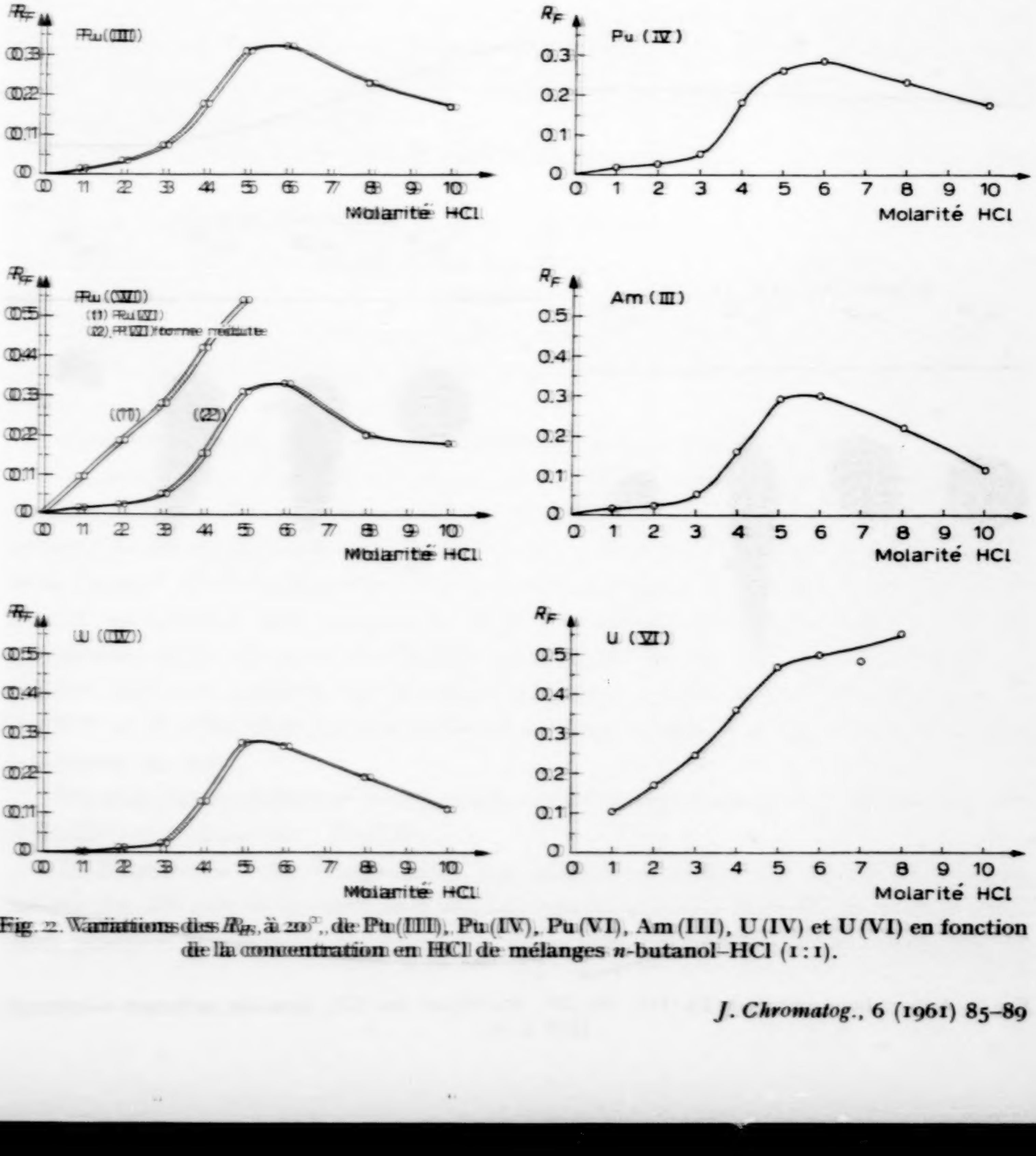

Fig. 2. Variations des R_F, à 20°, de Pu (III), Pu (IV), Pu (VI), Am (III), U (IV) et U (VI) en fonction de la concentration en HCl de mélanges *n*-butanol–HCl (1 : 1).

Résultats

Ils sont consignés dans le Tableau I.

Les courbes représentatives de variation du R_F en fonction de la molarité HCl pour les mélanges *n*-butanol-HCl (1:1) sont réunies à la Fig. 2.

L'examen des autoradiogrammes et des courbes d'activité des chromatogrammes que nous n'avons pas représentées ici montre:

(a) Pour Pu(III), l'existence d'un seul spot pour toutes les molarités de HCl.

(b) Pour Pu(IV), l'existence d'une seule tache jusqu'à la molarité 8 *M* de HCl; cette tache donne une petite comète pour les molarités HCl allant de 4 *M* jusqu'à 8 *M*; pour HCl 10 *M*, la tache Pu(IV) est, au contraire, précédée d'une longue traînée.

(c) Pour Pu(VI), on a deux taches très nettes jusqu'à HCl 5 *M*, puis aux molarités plus élevées, une seule tache précédée d'une longue traînée. L'examen du tableau des valeurs R_F permet de penser que le Pu(VI) est réduit à l'état IV (tache la plus près de l'origine) pour les molarités HCl inférieures à 4 *M*; pour les molarités HCl supérieures, il est difficile de conclure sur l'état de valence de cette forme réduite.

(d) Pour Am(III), on note, dans tous les cas, l'existence d'une seule tache.

(e) Pour U(IV) et U(VI), on note toujours une seule tache, le papier n'ayant aucun effet réducteur sur l'U(VI).

L'examen des courbes de la Fig. 2 montre une allure générale identique pour Pu(III), Pu(IV), Am(III) et U(IV) ainsi que pour la forme réduite du Pu(VI); U(VI) et Pu(VI) donnent des courbes similaires, comme on pouvait s'y attendre.

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Reçu le 20 mars 1961

The detection of ε-caprolactam on paper chromatograms

Until recently two direct methods have been used for the detection of caprolactam on paper chromatograms.

Zahn and Rexroth[1] identified caprolactam as dark blue spots when the paper had been subjected to direct chlorination and afterwards to the action of ammonia and *o*-tolidine in potassium iodide solution.

Czerepko[2,3], Franc[4], and Pavličkova[5] used potassium bismuth iodide solu-

tions for detecting caprolactam. This reagent gives red spots of complex caprolactam compounds on the yellow background of the paper.

It has also been found that caprolactam can be detected on paper chromatograms with iodine, potassium iodoplatinate[2,3], or with alcohol solutions of potassium antimony iodide[6].

In this report a new colour reaction of caprolactam is described which is the result of the action of an alkaline solution of *m*-dinitrobenzene.

Materials and methods. For one-dimensional ascending chromatography rectangular glass jars were used. Whatman No. 1 filter paper was employed.

Aqueous alcohol solutions were used to develop the chromatograms. The composition of the solvent systems is given in the previous publication[3]. The R_F values of caprolactam in these solvents was 0.8–0.9.

The chromatograms were dried in air. The following reagents were used for spraying:

(a) 1% methanolic solution of *m*-dinitrobenzene (*m*-DNB).

(b) 3 *N* methanolic solution of KOH.

Equal volumes of reagents (a) and (b) were mixed immediately before spraying the chromatograms.

Results. On the chromatograms, developed by means of the alkaline methanolic solution of *m*-DNB, caprolactam appeared in a few minutes in the form of intensive heather-coloured spots on the pale pink background of the filter paper. After 45 minutes the colour of the spots is deepest and after 2–3 hours the spots disappear.

The sensitivity of this reaction is about 5 μg of caprolactam. It is therefore similar to the reaction with potassium bismuthiodide in the modification that we employed[5], and hence it may be presumed that it could also be used for quantitative analysis.

The coloured compound obtained from caprolactam and *m*-DNB probably results from an alkaline condensation of the two compounds, a process similar to that occurring in the ZIMMERMANN[7] reaction where *m*-DNB also gives a violet-red colour reaction with 17-ketosteroids.

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Received February 28th, 1961

Notes

A note on the paper chromatography of some indole derivatives on acetylated paper

A very considerable interest has developed in the paper chromatography of indole derivatives in the past decade and there have been numerous publications on the subject (for lists of references see JEPSON[1] and BLOCK *et al.*[2]).

In these laboratories we have been concerned with two problems involving the paper chromatography of indole derivatives, firstly, the examination of the urinary indoles of mental patients and secondly, investigations into the chemistry of the aminochromes involving the necessity of obtaining suitable systems for chromatographing 5,6-dihydroxyindoles and indoxyls[3–5]. The difficulties involved in the former study, which are not entirely chromatographic, are well known and in the latter case the relative instability of many of the substances involved under the conventional conditions for indole chromatography instigated the investigation of the use of unconventional chromatographic systems for these compounds. The 5,6-dihydroxyindoles and their diacetyl derivatives can be separated very easily on formamide-treated paper with non-polar running solvents[5], but this type of system is not very satisfactory for the aminochromes or 5,6-dihydroxyindoxyls owing to the very low solubility of this latter group of compounds in non-polar solvents. Satisfactory R_F values for indole derivatives can often be obtained on formamide-treated paper with a suitable mobile phase[6,7]; with dichloromethane or benzene–ethyl acetate (3:1) as running solvent it is possible to effect certain separations that have proved difficult with the classical methods (*i.e.* (a) 3-indolealdehyde from 3-indoleacetonitrile and (b) 3-indoleacetic acid from 3-indolelactic acid)[7].

The use of acetylated paper, particularly for the separation of non-polar substances (such as the sugar acetates, aromatic hydrocarbons, etc., (*cf.* refs.[8–12]) has been known for some time, but as far as the authors are aware the use of this type of modified paper for studying the paper chromatographic behaviour of indoles has not yet been reported, and it was considered that such reversed-phase systems might offer certain advantages particularly when dealing with some of the relatively non-polar indoles.

This note reports the use of Whatman No. 1 paper (approximately 22% acetylated[9]) and the upper phase of a chloroform–methanol–water mixture (10:10:6), *cf.* ref.[13], as running solvent. A very good separation of a number of 3-indoleacetic acid derivatives could be obtained (see Table I): for instance, 3-indoleacetonitrile (R_F = 0.02) was easily separated from 3-indolealdehyde (R_F = 0.47). The hydrophobic indoles tested, such as indole itself, skatole, 3-indoleacetonitrile and ethyl 3-indoleacetate had relatively low R_F values (0.02; 0.08; 0.02 and 0.22 respectively) under these conditions, whilst the more polar substances tended to be relatively fast running.

This chromatographic system also appears to have certain potential uses when working with many of the substances encountered in studying aminochrome chemistry, *cf.* ref.[14] (see Table I), for instance adrenochrome ($R_F = 0.82$) was quite easily separated from two of its more common transformation products, adrenolutin ($R_F = 0.72$) and 5,6-dihydroxy-N-methylindole ($R_F = 0.37$); furthermore these substances seem relatively stable under these chromatographic conditions, in contrast to the ease with which compounds of this nature often decompose during chromatographic examination[3,4]. However, on drying the papers, after development with the solvent, the red adrenochrome spot slowly faded and was replaced by a yellow fluorescent spot, due to its isomerisation to adrenolutin (similar to the phenomenon observed on ordinary paper[3]).

The results obtained with fifteen different indole derivatives are given in Table I. The R_F values were found to be reasonably reproducible between different batches of paper. Although it might be considered desirable not to actually quote R_F values, but to refer the migration of a given substance to a standard substance each time, the actual R_F values obtained have been reported here to demonstrate the general order (which is always the same) of the mobility of the substances investigated.

TABLE I

R_F VALUES OF SOME INDOLE DERIVATIVES ON ACETYLATED PAPER*

Compound	*Average R_F value***	*Method of detection of spot****	*Source of compound*
5-Hydroxy-3-indoleacetic acid	71	A	Regis Chemical Company
3-Indoleacetic acid	57	A	Eastman Kodak Company
3-Indoleacetonitrile	02	A	Regis Chemical Company
3-Indolealdehyde	47	B	Regis Chemical Company
3-Indoleacetamide	67	A	Mann Research Laboratories Inc.
Ethyl 3-indoleacetate	22	A	Regis Chemical Company
Indole	02	A	Eastman Kodak Company
Skatole	08	A	Eastman Kodak Company
Tryptamine	04	A	Eastman Kodak Company
Lysergic acid diethylamide	04	A	Sandoz Pharmaceuticals (Canada) Ltd.
Adrenochrome	82	C	Prepared by the method of HEACOCK *et al.*[3]
Adrenolutin	72	C+	Prepared by the method of HEACOCK AND MAHON[4]
Adrenochrome monosemicarbazone	73	C	Labaz Company
5,6-Dihydroxy-N-methylindole	37	A	Prepared by the method of HEACOCK *et al.*[5].
5,6-Diacetoxy-N-methylindole	18	A	Prepared by the method of HEACOCK *et al.*[5]

* Using the upper phase of a chloroform–methanol–water (10:10:6) solvent system as running solvent.

** R_F values × 100.

*** A = Positive reaction (red to blue colours) with Ehrlich's reagent; B = Orange colour with 2,4-dinitropenylhydrazine; C = Self indicating (coloured substances); + = Exhibits a marked yellow-green fluorescence in ultraviolet light.

A few preliminary experiments were carried out with alternative running solvents including: (a) methanol; (b) various methanol–water mixtures and (c) various methanol–toluene–water (and related systems, *cf.* ref.[12]). However, with the current group of compounds, the separations achieved with these systems were not as good as with the chloroform–methanol–water system.

Experimental

Indole derivatives. The sources of the indole derivatives used in this investigation are given in Table I.

Paper. Whatman No. 1 paper was acetylated according to the method of MICHEEL AND SCHWEPPE[9]. The paper was immersed in a solution of acetic anhydride in benzene (1:3), containing 0.1% of concentrated sulphuric acid by volume; the mixture was heated, under reflux, at 70° for six hours. The acetylation mixture was decanted; the paper washed with cold water and then allowed to stand in methanol overnight. Finally the paper was soaked in water for three hours and air dried. It was washed for 24 hours with the running solvent, and dried, immediately prior to use.

Solvent system. A chloroform*–methanol*–water (10:10:6) system was prepared and allowed to stand at room temperature for 1 hour. The upper phase was removed and used directly.

Procedure. The chromatography was carried out at room temperature, in the ascending direction. A total rise of *ca.* 30 cm, taking *ca.* 5 hours was employed.

The investigations, which were supported by grants from the Government of Saskatchewan (Department of Public Health) and the Department of National Health and Welfare (Ottawa), are continuing and further results will be reported in due course.

Acknowledgement

The authors wish to express their thanks to Mrs. B. D. SCOTT for the preparation of several of the substances used in this investigation.

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Received March 24th, 1961

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* Reagent grade solvents were used.

An apparatus for applying reagents to paper chromatograms*

For the years I have worked with paper chromatograms I have attempted to apply the coloring agent evenly over the surface of the paper. I have been dissatisfied with all methods attempted until the construction of the apparatus described here (Fig. 1).

A 20 in. length of 2 in. diameter stainless steel tubing was cut lengthwise, and two quarters were opened out and flattened forming the trough and flanges, T. The upright ends, E, were milled from 1/2 in. steel and soldered to the trough. Two rollers were made, SR, one from 3/4 in. stainless steel bar stock, the other from 1/2 in. Lengths of 1 1/4 in. diameter foam plastic "Plasti-Paint-Rollers", FP, were forced over the 1/2 in. diameter bottom roller. The bottom roller dips into the trough of reagent, holding up a constant amount of reagent in the tiny pockets of the foam. The weight of the top steel roller compresses the foam slightly squeezing out a constant amount of reagent on the chromatogram. Each 18 × 22 in. chromatogram uses about 40 c.c. of ninhydrin reagent.

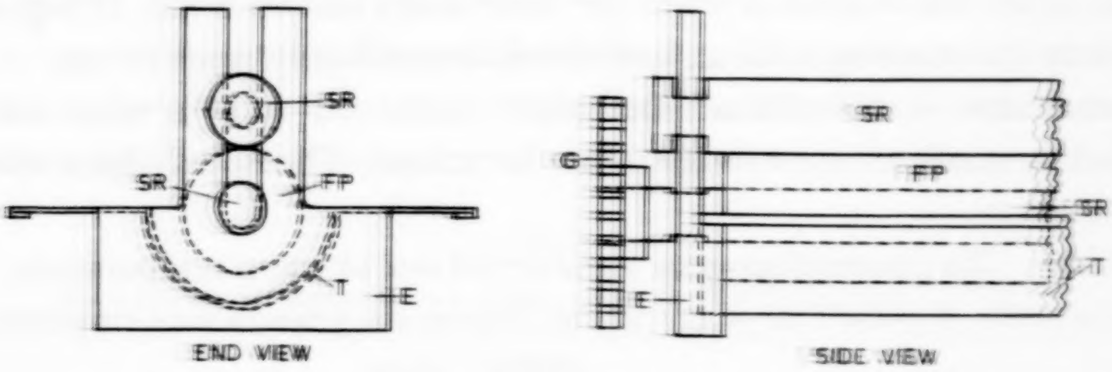

Fig. 1.

The bottom roller is driven by a reduction gear, G, and a small electric motor. The upper roller is driven by friction from the lower roller. The gearing is such that it takes but 30 sec. to run an 18 × 22 in. paper chromatogram through. This means that four chromatograms can be run in 2 min instead of the usual 15 to 20 min by hand spraying. This reduces the interval between the initial spraying and heating of a group of chromatograms, thus reducing the evaporation of an alcoholic coloring agent on the chromatogram to a minimum.

I wish to thank Mr. DONALD SIEMS of our shop, and my research assistant, Mr. WILLIAM SAUSEN for their skilful technical assistance in the construction of the apparatus.

Biological Sciences Department, H. EVERETT HRUBANT
Long Beach State College, Long Beach, Calif. (U.S.A.)

Received March 27th, 1961

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* This work was supported by a research grant RG-7314 from the Division of General Medical Sciences of the National Institutes of Health, U.S.P.H.S.

Solvent evaporator for paper chromatography

We use a device somewhat like that described by GANIS[1] for evaporating solvent during the application of solution to paper (Figs. 1 and 2). Our scheme differs in three respects whose description may be useful to others.

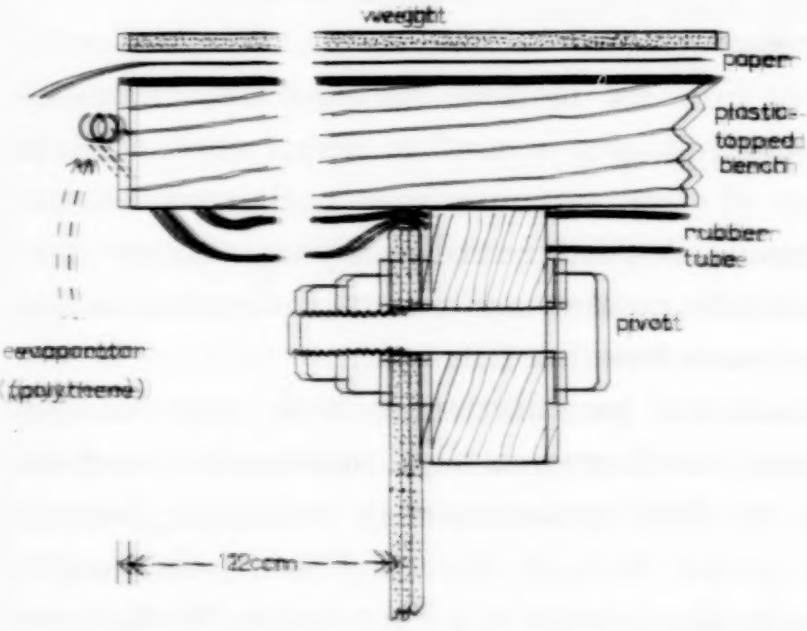

Fig. 1. Side view of evaporator, with clamping lever in closed position.

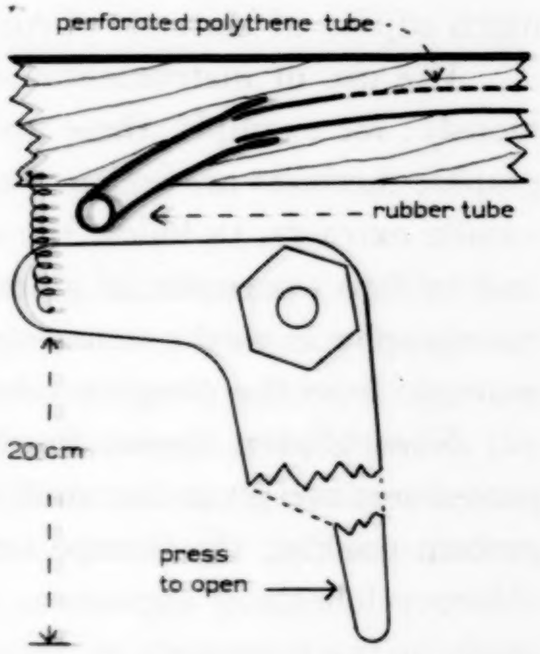

Fig. 2. Front view of lever system, showing "valve" in open position.

1. Polythene tubing is preferred to metal, because the holes are easily made with a hot pin. It is desirable, too, to avoid risk of accidental contact between paper and metal.

2. The N_2 is carried under the bench by a rubber tube against which a lever is held by a spring. The lever prevents N_2 flowing except when needed. Pressure from a knee releases the lever and allows gas to flow. Evaporation is thereby easily controlled. Alternatively a pedal may be used as described for control of a vacuum line for column chromatography[2].

3. A rectangular plate holds the paper on the bench. The paper overhangs the bench, so that the starting line comes over the perforated tube. This arrangement holds the paper against gentle pressure from the pipette during application of solution.

The device is used during the determination of tocopherol contents of leaves[3].

Dunn Nutritional Laboratory,
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V. H. BOOTH

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[1] F. M. GANIS, *J. Chromatog.*, 5 (1961) 84.
[2] V. H. BOOTH, *Carotene, its Determination in Biological Materials*, Heffer, Cambridge, 1957, p. 32.
[3] V. H. BOOTH AND A. HOBSON-FROHOCK, *J. Sci. Food Agr.*, 12 (1961) 251.

Received March 24th, 1961

BOOK REVIEW

Official Methods of Analysis of the Association of Official Agricultural Chemists, edited by WILLIAM HORWITZ, 9th Ed., The Association of Official Agricultural Chemists, Washington, D.C., 1960, xx + 832 pages, price \$ 17.50 (in U.S.A.); \$ 18.00 (elsewhere).

By increasing the page size, using two columns of type to a page and omitting the chapter on soils, the A.O.A.C. has managed to increase considerably the content of its ninth edition of Methods of Analysis and yet produce a manageable book.

The use of instrumental and chromatographic techniques has increased enormously; for example, there are now six infra-red methods included and chromatographic methods are legion. Paper chromatography is used to detect adulterants in vanilla extracts, to follow the efficiency of sugar separations on a charcoal column, and to detect commercial glucose in honey. Besides dyestuff analysis, column chromatography is used extensively in pesticide residue analysis to concentrate active material from the complex substrates always found in this work.

New chapters appear for disinfectants and for additives in feeds; new bioassay procedures are given for shell fish poison, antibiotics in feed supplements, and for protein quality; the isotope technique for BHC (mixed isomers of 1,2,3,4,5,6-hexachlorocyclohexane) appears as a First Action Method; the I.U.P.A.C. collaborative study on trace amounts of copper in foods also appears as a First Action Method and the molybdo-vanadate colorimetric method for phosphorus is introduced as an official method for certain fertilizers.

Although several new pesticide methods are given both for macro and residue analysis, the fact that there are methods given for less than one-fifth of the number of pesticides registered by the U.S.D.A. emphasises once more that much research is needed to find analytical methods of sufficient sensitivity and specificity for inclusion in this book.

The A.O.A.C. has maintained its usual high standard of book production and this volume is a worthy successor to its predecessors.

T. J. BECKMANN (Brisbane)

J. Chromatog., 6 (1961) 96

ERRATA

J. Chromatog., Vol. 5 (1961)

Page 199, line 7 from bottom: for "1/4 in." read "1/16 in.".
Page 200, legend to Fig. 3: for "C′ = Leveling bracket" read "C′ = Levering bracket".

THE DESIGN AND CONSTRUCTION OF A TWO-INCH PREPARATIVE GAS CHROMATOGRAPHIC COLUMN*

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(Received November 1st, 1960)

INTRODUCTION

Our interest in the field of acrylic and related monomers is, of course, well known. In this connection, it soon became apparent that a preparative gas column capable of producing pure samples of these monomers would be extremely useful. It further became apparent that the obtaining of these pure samples would greatly aid in the study and understanding of the reaction kinetics of polymerization. With these points in mind, it became almost a necessity either to purchase or design and construct a preparative gas column.

DISCUSSION

We have been interested in gas chromatography for approximately 5 years. Since our initial venture into gas chromatography, outstanding progress has been made in the development of the analytical gas–liquid chromatography instruments. The argon and flame ionization detectors and the capillary columns were developed to such a state that extremely small traces of material may be fully investigated. These methods are not amenable to sample isolation, and therefore, find use only in analytical investigations. It was felt that while these instruments were indeed a boon to the analytical investigations of odors and flavors, they are practically useless for obtaining workable quantities of pure samples. At the initiation of this project very few instrument companies had any suitable apparatus that would fit the job. Perhaps the first commercial preparative column was the 1-inch, 3-meter column designed by Perkin-Elmer Corporation as an accessory to their standard analytical instrument. Podbielniak has the Chromachron and very recently Beckman announced the sale of the Megachron. These are very fine instruments in themselves, but it was felt that the design and construction of our own column would give some insight into the cause and possible solution of some of the difficulties concerned with large scale gas chromatography.[1-3]

The design of the preparative gas column was based on a scale-up of our own "home made" analytical model, which consists of a 12-ft. 1/4-in. O.D. column, a Gow-Mac Thermal conductivity cell and the necessary power supply and recorder.

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* Presented at the second Delaware Valley Regional Meeting, February 25, 1960.

It was intended that a charge of 5 ml of total sample would be a suitable size to inject for separation. The figure calculated by scaling up from the 1/4 in. column, resulted in a preparative column of 2 in. diameter. The exact figure is less than 2 in. but it was decided that the extra size would allow 5 ml of sample to pass without unduly overloading the column. This figure was arrived at by the following calculations. The small column has an I.D. of 4.5 mm and a load limit of about 50 mg. To allow for 5 ml of sample, a column capable of handling 5000 mg should be used. The calcultations based on the ratio of the diameters squared,

$$\frac{x^2}{(4.5)^2} = 100, \text{ hence } x = 45 \text{ mm}$$

resulted in a column 45 mm in diameter. The capillary was chosen since the detection of samples in large volumes of high velocity gas streams has proven very difficult. The Gow-Mac thermal conductivity cell was chosen since it has been our experience that these units are quite sturdy, have excellent sensitivity over a wide temperature and are reasonably stable over long periods of time. It was also decided that the detector system would consist of a capillary by-pass and a Gow-Mac thermal conductivity cell. These were then the basis of the design and construction of a 2-inch column.

CONSTRUCTIONAL DETAILS

The column proper consists of a 2-in. I.D. stainless steel pipe, 9 ft. long, with the ends sealed with stainless steel caps (Fig. 1). One cap acts as a sample inlet and the other cap was modified to act as the outlet. A piece of 1/2 mm capillary stainless steel tubing acts as a capillary by-pass to the stainless steel detector cell (Fig. 2). All connecting tubing is either plastic or stainless steel.

Fig. 3 is a diagram of the inlet system. This was threaded onto one end of the column proper and is individually heated with a 6-ft. heating tape and powered by a Variac. The temperature is maintained approximately 50° above column temperature to insure rapid and complete vaporization of the sample.

The stainless steel helices used to pack the inlet cap weigh approximately 100 g, which was considered sufficient to insure good vaporization without any appreciable cooling of the injection block.

Fig. 4 is a diagram of the outlet and condensing systems used for the recovery of the separated fractions. At present, there is only one trap since we are mainly concerned with the isolation of the main constituent. More could be added without any difficulty.

The detector cell is a Gow-Mac TE-II design; stainless steel, hot wire filament powered by a Gow-Mac power supply. The cell is housed in a specially designed unit which consists of a large Dewar flask and a heating unit. This unit plus the outlet system are heated individually with their own power supply and are maintained approximately 50° above column temperature. The output of the cell is connected to a 0.5 mV strip-chart recorder running at a chart speed of 6 in. per hour.

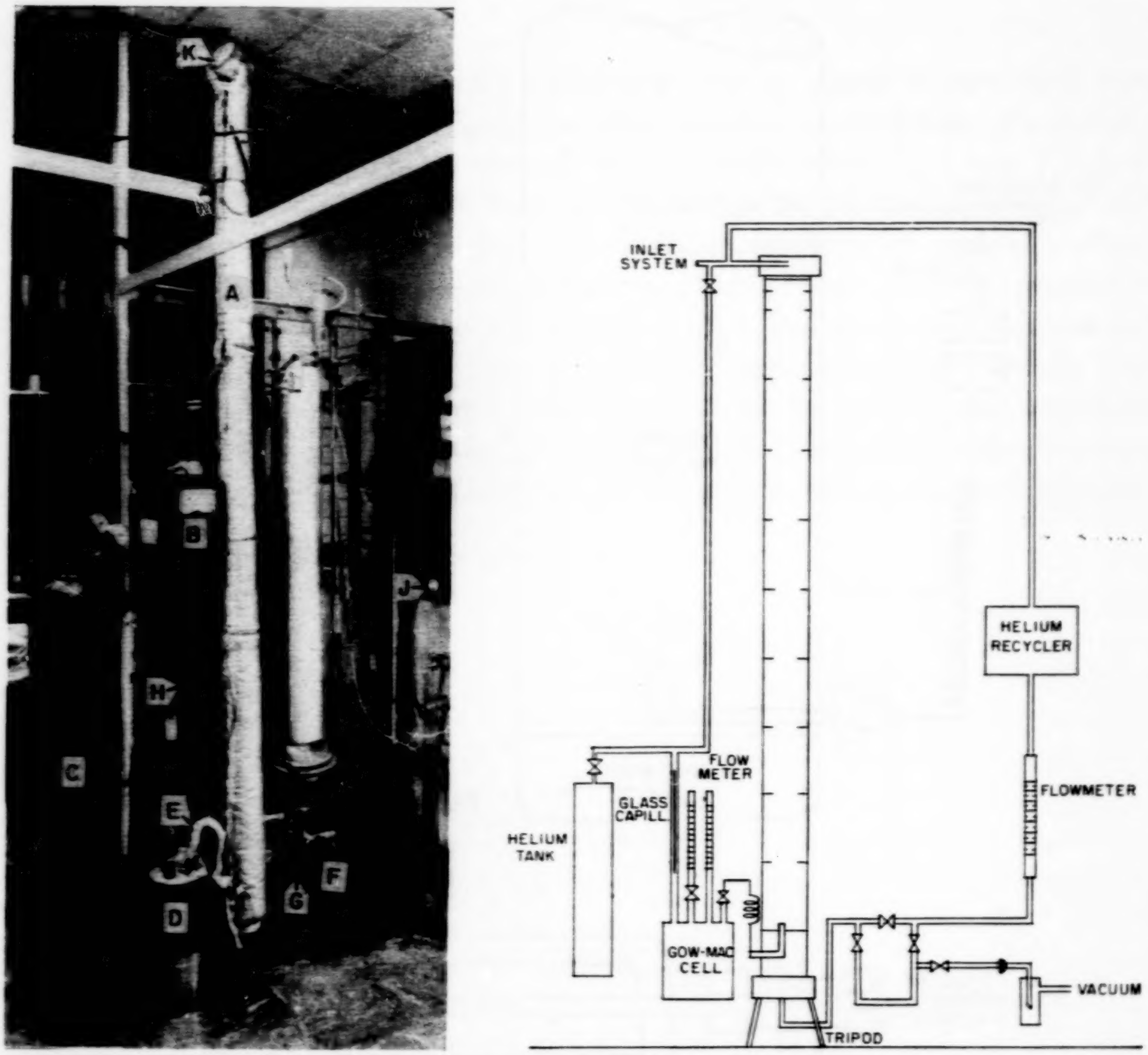

Fig. 1. Two-inch gas preparative column. (A) Column proper showing heating mantles. (B) Multipoint thermocouple meter. (C) Helium cylinder. (D) Detector and housing. (E) Capillary by-pass. (F) Helium recycler. (G) Outlet and condensing system. (H) Flow meter for capillary flow control. (J) Detector power supply. (K) Sample inlet.

Gas circulating system

The original column was set up to use nitrogen gas but the rapid rate of flow very soon depleted the cylinder. The annoying task of replacing a new cylinder every morning, was soon alleviated by a helium gas recirculating system designed and built by our Instrument Laboratory. This unit consists of 2 pressure gauges, 3 traps, 4 valves, a compression pump and a surge tank. In operation, the helium cylinder gauge is set such that the circulator high pressure gauge will register at 15 lb./in.^{2}. The second gauge is set by valve at 15 in. of water. The purpose of the second gauge and valve is to by-pass some of the gas from the high side of the compressor to prevent a vacuum on the outlet side of the column. The four valves are used to control the inlet, the outlet and by-pass stream, and also as a vent to flush contaminants out of the apparatus. The 3 traps are made of brass and are filled with activated charcoal, dehydrite,

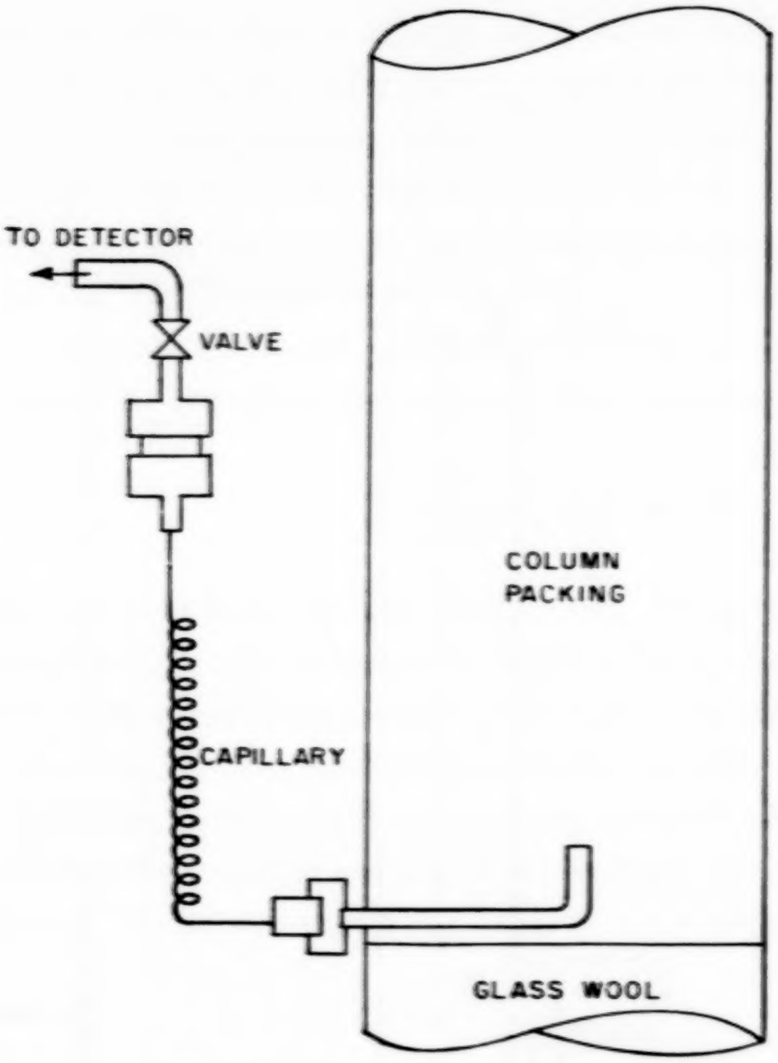
TO DETECTOR
VALVE
COLUMN
PACKING
CAPILLARY
GLASS WOOL

Fig. 2. Capillary by-pass system.

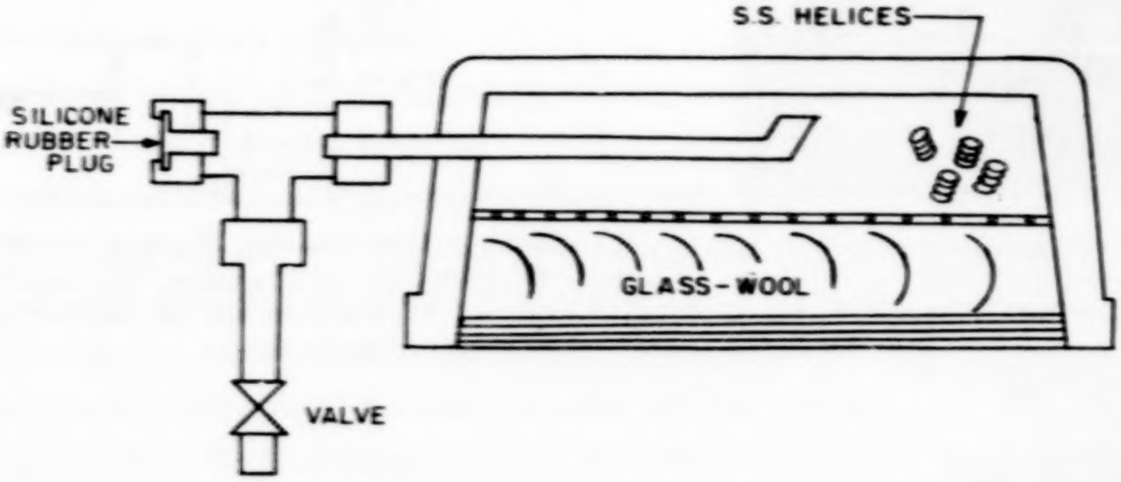
S.S. HELICES
SILICONE
RUBBER
PLUG
GLASS-WOOL
VALVE

Fig. 3. Sample inlet system.

and ascarite, respectively. During operation of the column, these traps are immersed in a dry-ice acetone bath. To be absolutely certain that no contaminants re-enter the gas stream, a 4th trap of molecular sieves was separately added just previous to the main traps. This trap is continuously immersed in liquid nitrogen during operation and is quite useful since it will rapidly condense and hold most of the contaminants thereby facilitating clean-out. It is essential that these traps be purged at least once a week if the column is used heavily, since any sample that is not adsorbed by the trap will be recirculated and thereby show up as an impurity in the next fraction collected. Of course, this problem will change according to the demands put to the column.

Condensing system

The condensing system consists of 4 valves and a 1/2 in. copper U-tube filled with stainless steel helices (Fig. 4). During operation, previous to condensing the sample, the carrier gas stream is passed through valve 1, while valves 2, 3, and 4 remain closed. During condensation the U-tube is immersed in dry-ice acetone, valve 2 and valve 3 are opened and valve 1 closed. The point to condense the sample is determined by the appearance of the peaks on the strip-chart recorder. After the sample has passed, valve 1 is reopened and valves 2 and 3 are closed. The system is then evacuated by opening valve 4 and valve 5. The dry-ice bath is removed and the receiving trap immersed in liquid nitrogen. As the U-tube warms to room temperature, the condensed sample will be transferred to the receiver trap. Finally, a hot water bath is placed around the U-tube to complete the transfer of sample from the U-tube to the receiving trap.

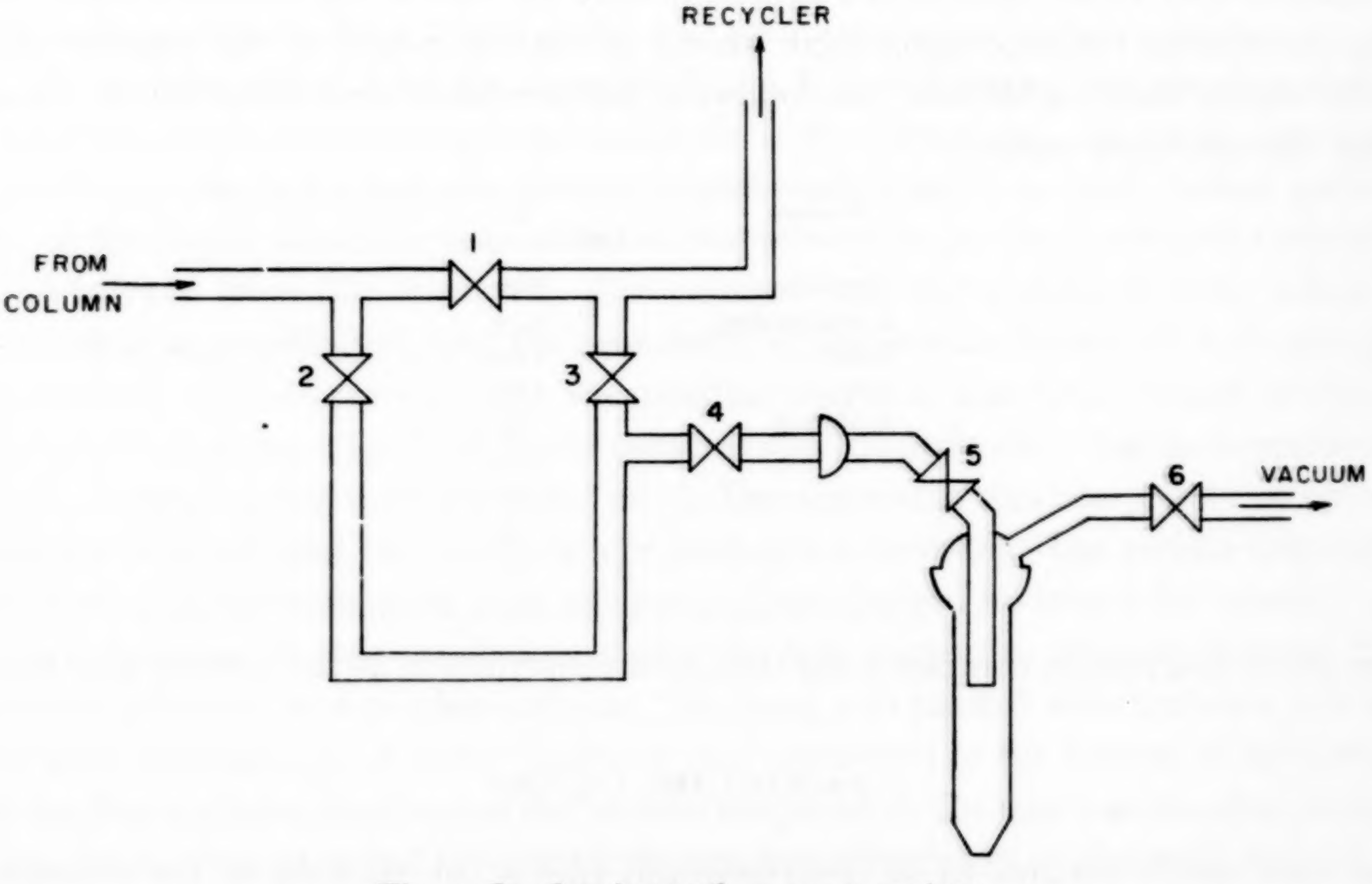

Fig. 4. Condensing and recovery system.

The efficiency of the trap represents about 95 % recovery, based on the recovery obtained when 3 ml of benzene was injected. It was necessary to design this type of condensing system since the entire apparatus is essentially closed and any direct condensing system without subsequent transfer might seriously disturb the equilibrium of the entire system.

Gas flow and pressure regulation

The gas flow and pressure regulation of the entire apparatus is controlled by the gas recycling system. The pressure in the preparative column is maintained at 15 lb./in.^{2}

by the compressor and a flow of 4.5 on the predictability flow meter, which corresponds to 1200 ml/min of He, is regulated by a valve on the helium recycler. Approximately 15 in. of water is maintained on the recycler by-pass valve situated on the high side of the pump for the reason explained above. To maintain a constant flow of 75 ml/min of gas through the sample side of the detector cell, a down-stream differential flow controller was used. A system of valves and a flow meter also control the flow of 75 ml/min of gas through the reference side of the detector cell.

Temperature control

There is no elaborate or precise temperature control in the entire system, although reasonable precaution was taken to insulate the entire apparatus from rapid temperature changes. The column proper is heated to 70° by 3 Glas-Col mantles wired in parallel and powered by a 15-A powerstat. The inlet system, outlet system, condensing system, and detector cell are individually heated by heating tapes controlled by powerstats and all are maintained approximately 50° above the column temperature. Iron constantan thermocouples were placed at various points of the apparatus and under steady state conditions, the following temperatures were recorded on a multipoint thermocouple meter:

Position		*°C*
Outlet		130
Condensing		125
Inlet		120
Cell		155
Column	1	65
	2	75
	3	70
	4	75
	5	75

The above description completes the details of construction as the column now stands.

PACKING THE COLUMN

One final detail remains to be described and this is the packing of the column. At present the column is packed with approximately 5 lb. of 20% N,N-bis-(2-cyanoethyl)-formamide liquid phase on 30–60 mesh crushed firebrick. The above liquid phase was developed in conjunction with Dr. NEWMAN BORTNICK. It was selected for a very special purpose but has proven to be extremely useful in many other types of separation. It has an excellent column life even to approximately 125°. It is expected that a brief note describing the characteristics of this liquid substrate together with other liquids developed in our laboratory will soon be submitted for publication. By far the most difficult task of the entire project was the packing of the column. Many different methods were tried in an attempt to obtain a reasonably efficient column. A brief description of these methods together with some of the reasons for trying them follows.

The first method of packing was merely pouring in the packing with simultaneous vibration while the column was connected to a vacuum pump. The column was vibrated until no more settling was noticed. This method was tried initially since this is the method used on our analytical columns and appeared to give reasonably good results. The column was then set up and put into operation. The first sample through the column was a sample of *n*-hexane. The peak from this first sample was very broad and indicated that the column was operating very inefficiently. The column was then shut down and another method of packing tried. The second method was to pour in approximately 250 ml aliquots of packing and then tamping with a specially designed tamp. The complete operation was performed with a vacuum pump on the outlet side until the entire column was full. The reason for this method was that if the column was packed in discrete sections, any channelling that might occur in the previous method of packing would now be isolated to one small section of the column. It seemed unlikely by this method of packing that the entire length of the column would channel in the same direction. The results of this method were also very poor as indicated by broad peaks and poor separation between peaks.

The third method of packing was by slurry. It is well known that the best method of packing liquid chromatographic columns is by slurry and, therefore, by reason of analogy a slurry-packed gas column might work equally as well. Before packing, 1 lb. of the liquid substrate was added to the solvent to prevent complete removal of the substrate from the firebrick. The column was slurry-packed with *n*-hexane, all the drainings collected, and the remainder of the hexane blown off with nitrogen. The solvent was evaporated and the residue weighed. The final weight of residue was 1.25 lb., leaving 0.75 lb. of liquid substrate on the firebrick. This then represented a 15 % column rather than the usual 20 %. The apparatus was completely assembled, a sample injected and the resulting chromatogram recorded. The results were again very discouraging indicating poor efficiency characterized by broad flat peaks.

At this time, it was decided to study the flow properties of gases through large diameter columns. A 2-in. glass column, 3 ft. long, was packed with firebrick and then saturated with hexane. A water aspirator was connected to the bottom of the column and the flow pattern observed as the hexane evaporated. The flow pattern observed was in the shape of an inverted bowl with the gas traveling faster at the walls than at the center. It was apparent then that if the samples were traveling down the column as inverted bowl shaped bands, the recorded curve should be broad and have overlapping peaks even though good separation may have been accomplished. The reason why the peaks would overlap is shown in Fig. 5A. During the preliminary experiments the capillary by-pass was connected to the outlet side of the column so that the entire sample had to pass through the outlet before being detected. If the capillary were placed at the center of the packing, such that only the dome section of the bowl passed the capillary system, the recorded curves should approach those of an analytical column (Fig. 5B). To test this approach, the column was dismantled and the capillary fitting added to the column so that the entrance to the by-pass was exactly in the center of the packing at the bottom of the column. The column was repacked, and a sample of benzene and toluene introduced. The resulting curves showed a very marked improvement. The difficulty of this arrangement, however, was the fact that the recorded curves did not truly represent the samples as they emerged from the column. This discrepancy was found by connecting the capillary by-pass first to the center of the packing and then to the tubing on the outlet side. The curves from the center capillary showed definite peak separation, whereas the curves obtained by the capillary connected to the outlet side were overlapped and broad. It was apparent then that the assumption that these bands were moving as inverted bowls was reasonably correct. Once again, the column was dismantled and the packing removed. This difficulty brought to mind the discussion of GOLAY[1], who expressed the thought that "mixing washers" would be necessary for the efficient operation of large columns. With this thought in mind, the following experiment was attempted.

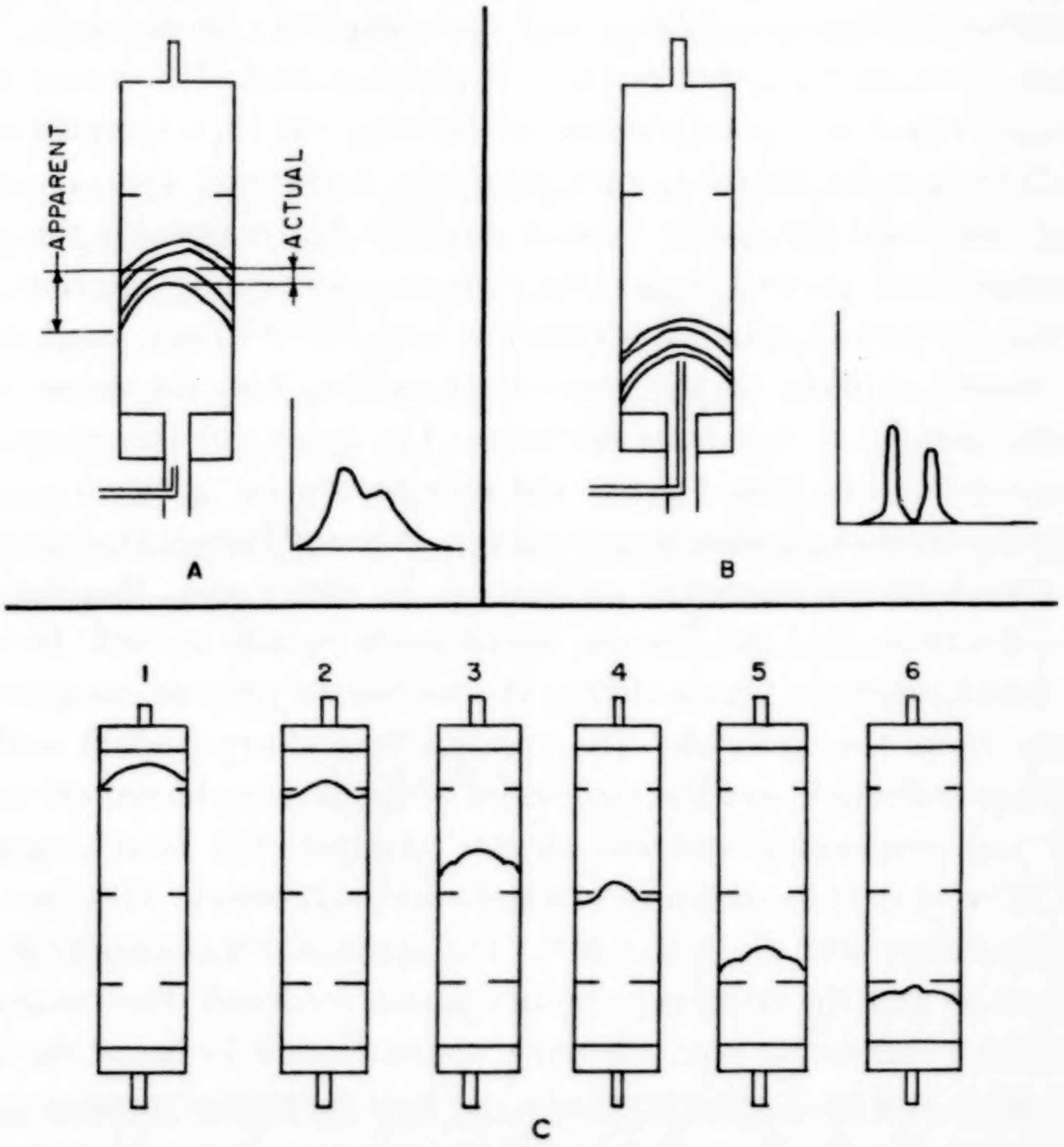

Fig. 5. Flow pattern.

Filter paper rings were cut to the exact size of the column and then saturated with substrate. These rings were graduated in size starting with a ring 2 in. O.D. and 1 7/8 in. I.D. and ending with a ring 2 in. O.D. and 1 3/8 in. in I.D. The rings were spaced 1 ft. apart along the length of the column. The column was packed by filling with vibrating to a depth of 1 ft. and then adding a saturated ring, another foot of packing and then another ring with an opening smaller than the previous ring, the largest aperature ring at the top and the smallest aperature ring at the bottom. After the column was packed in the above manner, the apparatus was completely reassembled and put into operation. Fig. 5C depicts a series of drawings illustrating my conception of the flow pattern of the sample bands as they pass over these rings. It is felt that since the carrier gas is flowing more rapidly along the walls of the pipe, a packing containing a homogeneous concentration of substrate will cause the bands to travel as an inverted bowl. If, however, it were possible to put a concentration gradient of liquid substrate across the radius of the column, then the sample bands should be more strongly adsorbed at the walls than at the center and, therefore, the sample bands should at least approach a straight line. Lacking suitable means of putting a concentration gradient across the column, the concept of saturated rings was employed.

The first drawing, Fig. 5C (1), shows the sample going on the packing. As the sample travels down the column, the bands start to distort, Fig. 5C (2). Passing the first and largest aperture, the ends of the bands are retarded slightly since there is a higher concentration of substrate even though the carrier gas is moving more rapidly at the sides than at the center of the packing. As the sample travels down the column, Fig. 5C (3–6), the bands become more distorted and will, therefore, require a greater amount of substrate at the wall to correct the distortion. This was the reason why the rings were graduated in size starting with the largest aperture near the inlet and going to the smallest aperture near the outlet.

When the column reached equilibrium, the previous experiment of connecting the capillary to two positions was repeated. A sample of benzene and toluene was injected and as was expected, the recorded curves from the center capillary showed good peak shape and high resolution. The capillary was then connected to the outlet tubing and another sample injected. The chromatogram obtained was very gratifying indeed. The peak shape was very good and the resolution was also very good. A calculation of the theoretical plate efficiency showed about 500 plates.

EXPERIMENTAL

Once the point had been reached where the column was shown to be reasonably efficient, a systematic investigation of the effects of the various parameters of gas flow, pressure, and sample size on the efficiency of the column was begun. Table I is a summary of the study of theoretical plate efficiency *versus* the flow rate through the column at 15 lb./in.^{2} at the inlet and at a constant cell flow rate through the detector. Table II is a study of the efficiency *versus* the flow rate through the detector at constant column flow rate. The operating conditions based on the above experiments were finally set at 15 lb./in.^{2} a flow rate of 1200 ml/min through the column and 75 ml/min through the cell. Table III is a study of the efficiency *versus* pressure at a constant flow rate through the cell and column. Table IV shows the changes in efficiency and separating power *versus* sample size. Table IV also indicates that even though the theoretical plate efficiency decreases rapidly, the separating power of the substrate remains fairly constant.

TABLE I*

VARIATION OF THEORETICAL EFFICIENCY PLATES WITH VARYING FLOW RATES

(Detector flow rate constant at 75 ml/min)

	Flow rate ml/min	*Theoretical efficiency (plates)*
2	270	284
3	590	395
4	1000	540
5	1450	459
6	1900	454
7	2360	323
8	2820	242

* 0.5 c.c. benzene.

TABLE II*

VARIATION OF EFFICIENCY WITH VARYING FLOW RATES THROUGH THE DETECTOR

(Column flow rate constant at 1200 ml/min)

Flow rate ml/min	*Theoretical plates*
20	522
41.3	522
58.0	496
80.0	526
300.0	506
480	560

* 0.5 c.c. benzene.

TABLE III*

VARIATION OF EFFICIENCY WITH VARYING HELIUM PRESSURE AT INLET

(Column and detector constant at 1200 ml/min and 75 ml/min respectively)

Per cent/in^2 at inlet	*Plates*
5	430
10	498
15	526
20	496
25	472

* 0.5 c.c. benzene.

TABLE IV

VARIATION OF PLATES AND SEPARATING POWER WITH SAMPLE SIZE (BENZENE AND TOLUENE) AT CONSTANT PRESSURE (15 lb./in.^{2}) AND CONSTANT FLOW RATE (1200 ml/min)

Sample size (c.c.)	*Sensitivity (%)*	*Separating power**	*Plates*
0.2	15.0	1.65	615
0.4	7.5	1.66	595
0.8	3.75	1.66	515
1.6	1.88	1.65	332
3.2	0.94	1.66	256
5.0	0.47	1.65	178

* $\dfrac{rt_2 \textit{ vs.} \text{ air}}{rt_1 \textit{ vs.} \text{ air}}$

in which rt_2 = retention time of peak 2 from air peak; rt_1 = retention time of peak 1 from air peak.

RESULTS

Figs. 6–9 are typical chromatograms obtained with the 2-in. column. Fig. 6 is a chromatogram of ethyl, *n*-propyl, and *n*-butyl acetate. Fig. 7 is a sample of aromatics and Fig. 8 is a sample of alcohols. Fig. 9 is a chromatogram of a sample of 2B alcohol.

Sensitivity

As seen from the chromatogram of 2B alcohol the maximum sensitivity of the apparatus is 3400 divs./ml of benzene. Under steady state conditions, short term noise is ± 1.5 divs. at 100% sensitivity. Long range noise is 1 div./h at 10% sensitivity.

Cost

A brief rundown of the cost of the various items used in the construction of this column are listed below:

9 ft. of 2-in. stainless steel pipe	$ 75.00
2 stainless steel caps at $ 6.50 each	13.00
4 stainless stop-cocks at $ 6.00 each	24.00
Assorted stainless steel tubing	10.00
1 Gow-Mac cell	65.00
1 Dewar	15.00
1 Power supply and att. (for cell)	100.00
1-20 A powerstat transformer	50.00
3 Glas-Col mantles each $ 65.00	195.00
3 Flow meters	30.00
5 lb. firebrick	60.00
Miscellaneous	25.00
Helium recycler	200–250.00
3–7 A powerstats	55.00

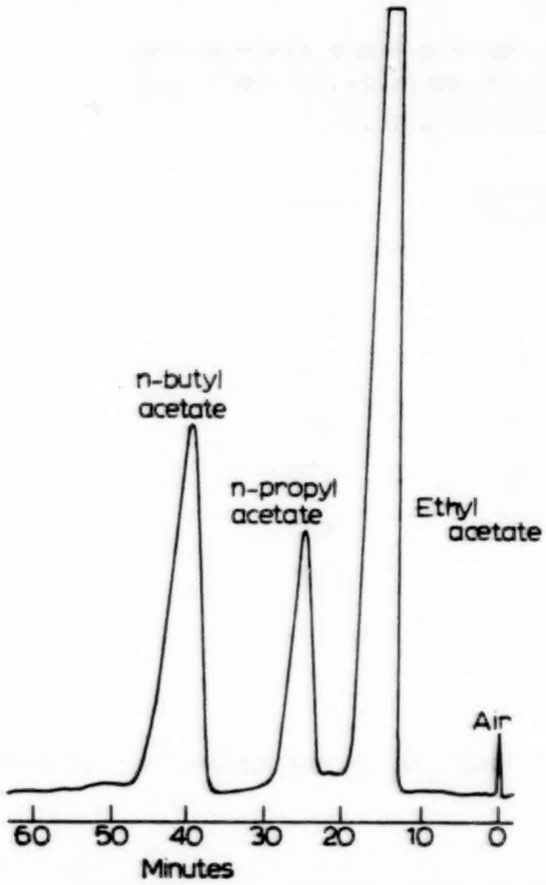

Fig. 6. Chromatogram of ethyl, *n*-propyl, and *n*-butyl acetate.

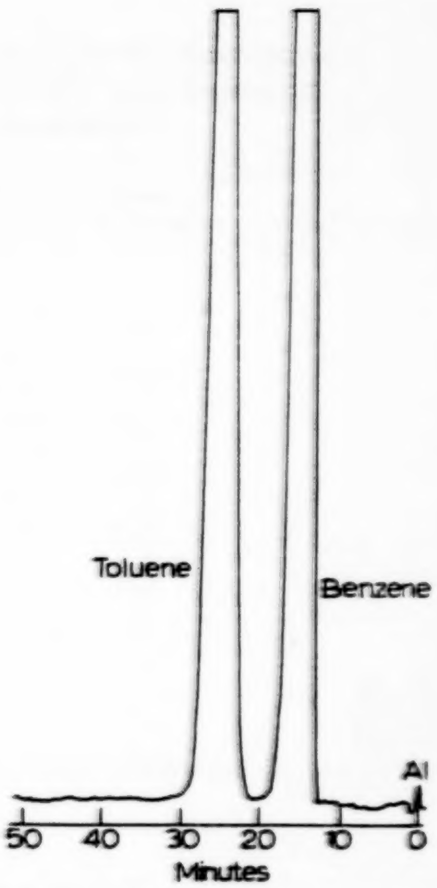

Fig. 7. Chromatogram of a sample of aromatics.

2-in. prep. column. Packing: N,N-bis-(2-cyanoethyl)-formamide. Flow: 1200 ml He/min (column); 75 ml He/min (capillary). Temp. 70°. Sensitivity: 10% on 0.5 mV F.S.D. Sample 2 ml. Pressure: inlet 15 lb./in.^{2}; outlet 12 lb./in.^{2}. Bridge current 150 mA.

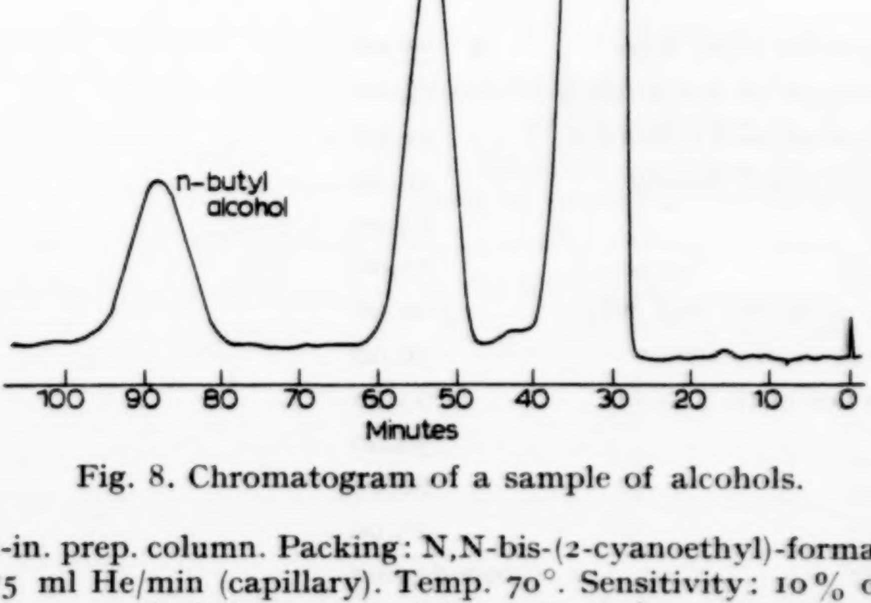

Fig. 8. Chromatogram of a sample of alcohols.

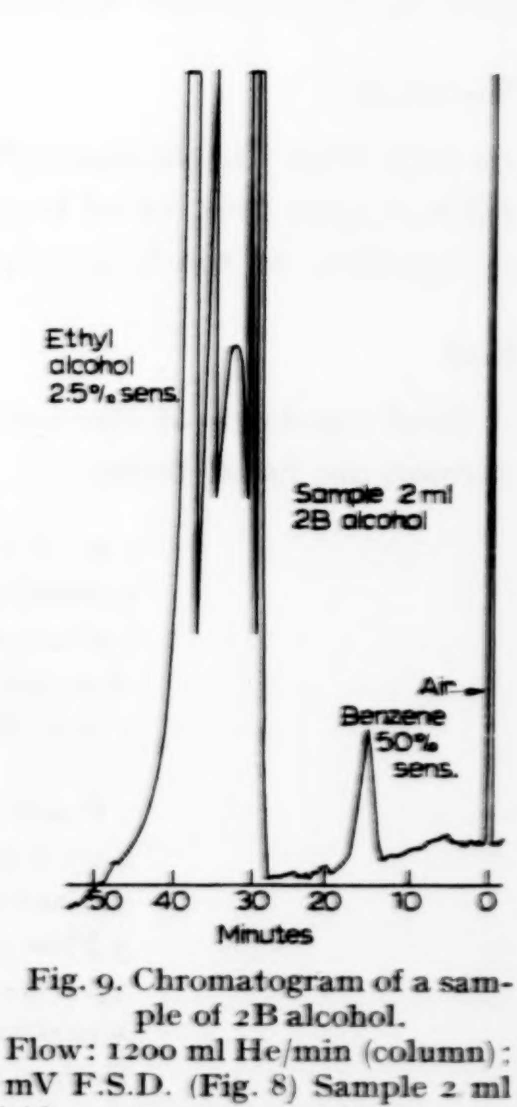

Fig. 9. Chromatogram of a sample of 2B alcohol.

2-in. prep. column. Packing: N,N-bis-(2-cyanoethyl)-formamide. Flow: 1200 ml He/min (column); 75 ml He/min (capillary). Temp. 70°. Sensitivity: 10% on 0.5 mV F.S.D. (Fig. 8) Sample 2 ml alcohol. Pressure: inlet 15 lb./in.^{2}; outlet 12 lb./in.^{2}. Bridge current: 150 mA.

CONCLUSION

The design and construction of a 2-in. gas chromatographic column has been described in full detail. A method of eliminating the wall effect due to large diameter packing has been extended and suitable experimental data to partially substantiate the proposal has been presented. The proposal has by no means been rigorously proven but is only extended as a possible explanation of the observed experimental data. This column has currently been in use for the past year and has performed quite efficiently in a wide variety of situations.

ACKNOWLEDGEMENTS

My sincerest thanks to my fellow co-workers for many suggestions and especially to Mr. DONALD GREENWAY, my assistant, who so patiently packed and unpacked this column so many times and to our instrument group for the helium recycler.

SUMMARY

Factors which control the design of a large column, such as column size, sample size, carrier gas flow, temperature, injection system, and condensing system are discussed. Other points of interest such as capillary by-pass and sample detection, packing problems, gas recycle, study of efficiency *versus* flow rate and efficiency *versus* sample size are also treated. A brief survey of the cost of materials is given.

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LINEARITY AND RESPONSE CHARACTERISTICS OF THE FLAME IONISATION DETECTOR

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(Received November 7th, 1960)

INTRODUCTION

The application of gas chromatography to the analysis of both major and minor components in a sample requires either a detector of high sensitivity and wide response range or a system involving either sample splitting or preconcentration techniques. A high sensitivity detector is more rapid and much simpler. If, however, such a detector is used, it is necessary to know whether its response is strictly linear over the required range. Most detectors are linear over a range of less than 10^4 to 1. Recent reports[1–4] on the linearity of the flame ionisation detector[5,6] have indicated a linear range of 10^7 to 1 and up to 0.5 % constituent in the gas stream. As part of a study of the response characteristics of this detector, its upper linear range and optimum operating conditions have been investigated in some detail at the ICIANZ Central Research Laboratories.

APPARATUS

The detector construction was similar to that described previously[5], but using a single jet and an electrical bias to offset the background current at high sensitivity. A single valve (ME1403) impedance conversion circuit with a gain of 1/15 and grid input resistors of 10^5–10^{11} ohms were used. Gas flow rates were measured in terms of the pressure drop (5–35 p.s.i.g.) across glass capillaries calibrated with a soap film or moving bubble flow meter. The air flow rate in the 9 cm diameter combustion chamber (containing two jets) was approximately 1 litre/min, and the data shown were obtained with a flat probe (upper) electrode consisting of a 1.5 cm square of approximately 25 mesh BSS brass gauze and a jet made from 20 gauge hypodermic needle tubing.

RESULTS AND DISCUSSION

Effect of applied voltage

The detector output for a given gas concentration increaces with the voltage applied between the probe electrode and the jet until a plateau is reached. It is assumed that the plateau corresponds to collection of all ions formed in the flame. For an impurity flow rate of 50 μg/sec and a probe to jet distance of 5 mm (see below) the plateau was reached in this investigation at approximately 360 V and extended at least to above 900 V. For lower amounts of impurities, *i.e.* smaller number of ions, lower voltages are adequate as shown in Fig. 1. This effect is due partly to greater ion recombination at high concentrations and partly to space charge effects. It is seen that a considerably lower voltage can be used on equipment designed solely for work with capillary columns[3,7] than for general purpose units for use with granular packed columns and larger samples.

At low concentrations a positive probe electrode gives superior ion collection efficiency to a negative probe, as would be expected from the higher mobility of electrons compared with positive ions. However, at higher concentrations, provided that the probe–jet gap is not too large, better results are obtained with a negative probe (see Fig. 1).

Effect of probe to jet distance

If a flat probe electrode is used, the efficiency of ion collection is markedly influenced by the distance between the probe electrode and the tip of the flame jet. Examples of this effect are given in Figs. 1, 2 and 3. Fig. 2 shows that at higher concentrations the probe–jet distance is very critical if linearity is to be obtained, while Fig. 3 indicates that even at the lower concentrations a very wide gap should be avoided. A hat-shaped collector electrode consisting of a flat upper section and a cylinder surrounding the flame, so that a large solid angle is subtended at the jet by the probe electrode, gives results similar to the small probe–jet gap without overheating the probe electrode.

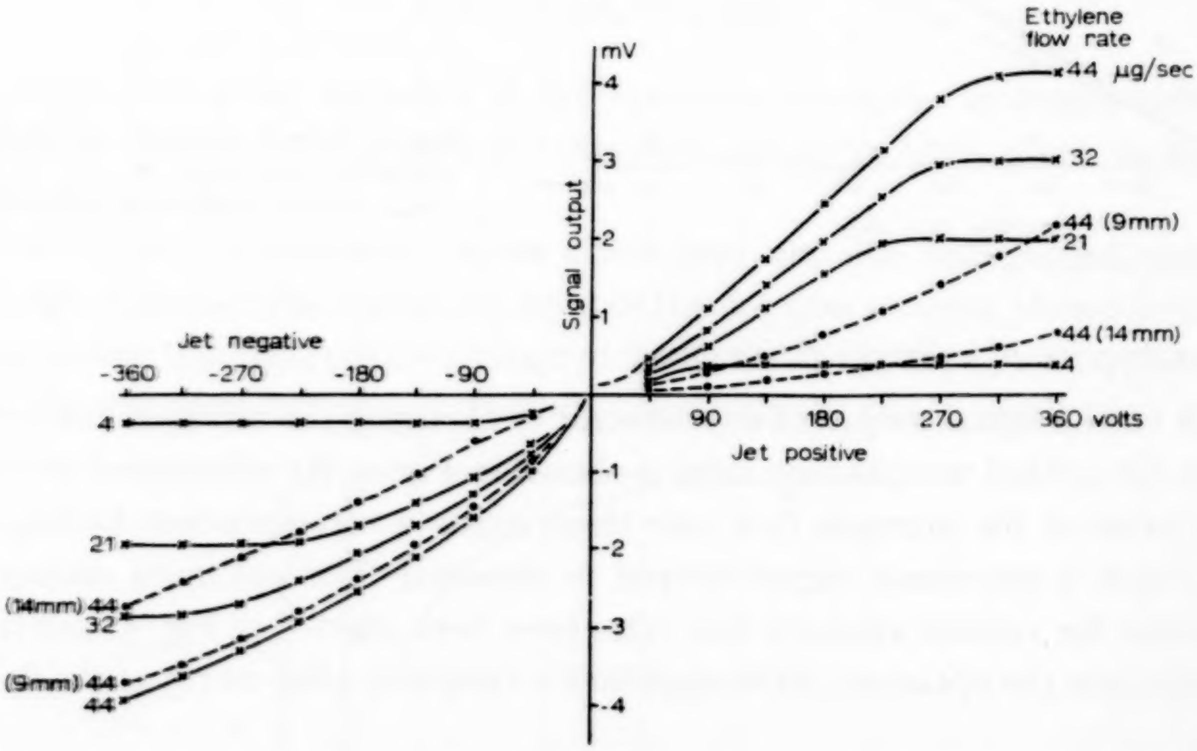

Fig. 1. Effect of probe–jet voltage on ion collection efficiency (grid resistor 1 × 10^5 Ω nominal, nitrogen flow rate 38 ml/min, hydrogen flow rate 38 ml/min, probe–jet distance 5 mm or as stated).

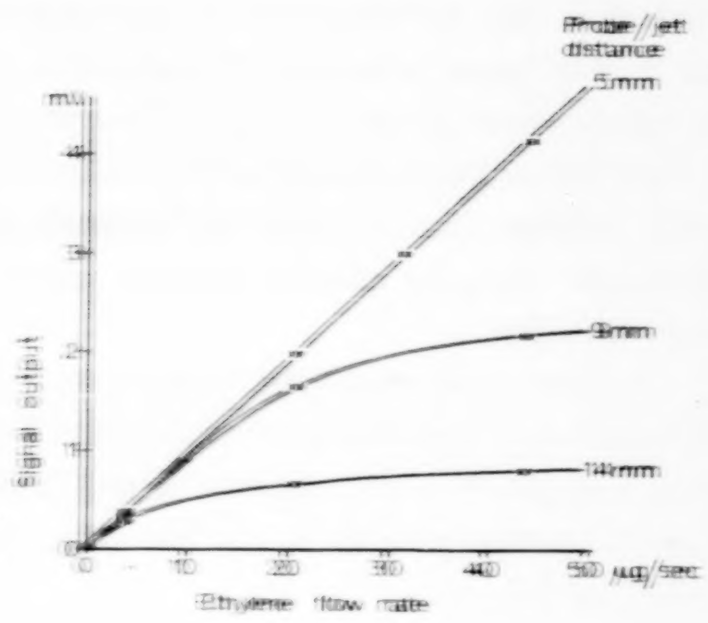

Fig. 2. Effect of probe–jet distance on linearity (conditions as for Fig. 1, probe–jet voltage 360 V, jet positive).

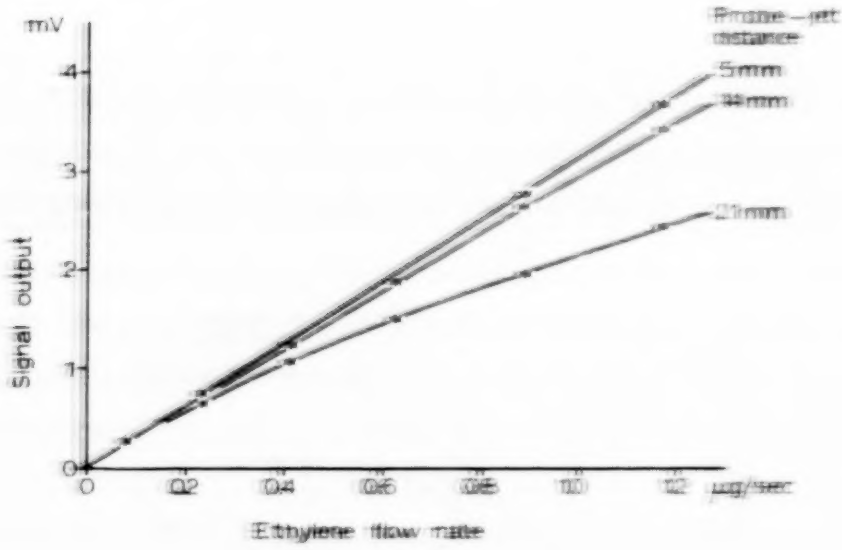

Fig. 3. Effect of probe–jet distance on linearity (grid resistor 3 × 10^6 Ω nominal, other conditions as for Fig. 2).

Effect of nitrogen and hydrogen flow rates

The effect on the signal output of the detector of changing the nitrogen and hydrogen flow rates (at a fixed sample flow rate) is shown in Fig. 4. As mentioned previously[5], for each value of the nitrogen flow rate there exists a corresponding hydrogen flow rate at which a maximum signal output is obtained. The optimum nitrogen : hydrogen ratios for various nitrogen flow rates have been plotted in Fig. 5, and it is seen that in this case the optimum ratios approach a constant value of 1.5 as the flow rates increase.

The optimum signal output conditions appear to correspond to those for the maximum signal to noise ratio of the detector. However, at any fixed nitrogen flow rate, as the hydrogen flow rate is increased past the optimum value, the signal not only decreases but the total background appears to increase almost exponentially. For this reason, and since the change in background ion current with change in nitrogen or hydrogen flow is dependent on the nitrogen and hydrogen impurity concentrations under non-optimum flow conditions (see Fig. 4), ultimate sensitivity determinations by the hydrogen addition method used by ONGKIEHONG[2] must be done under optimum flow conditions. The difference between the optimum ratio found in this study and that reported by CONDON, SCHOLLY AND AVERILL[1] is presumably due to their use of a thick-walled jet to serve as a heat sink, since the ratio appears to be relatively independent of the internal diameter of the jet.

Examination of Fig. 4 shows that the linearity of response is maintained for all nitrogen and hydrogen flow rates under conditions of efficient ion collection. It should also be noted that when operated at the optimum nitrogen : hydrogen ratio, small changes in the hydrogen, nitrogen or total flow rate have little effect on the signal output of the detector.

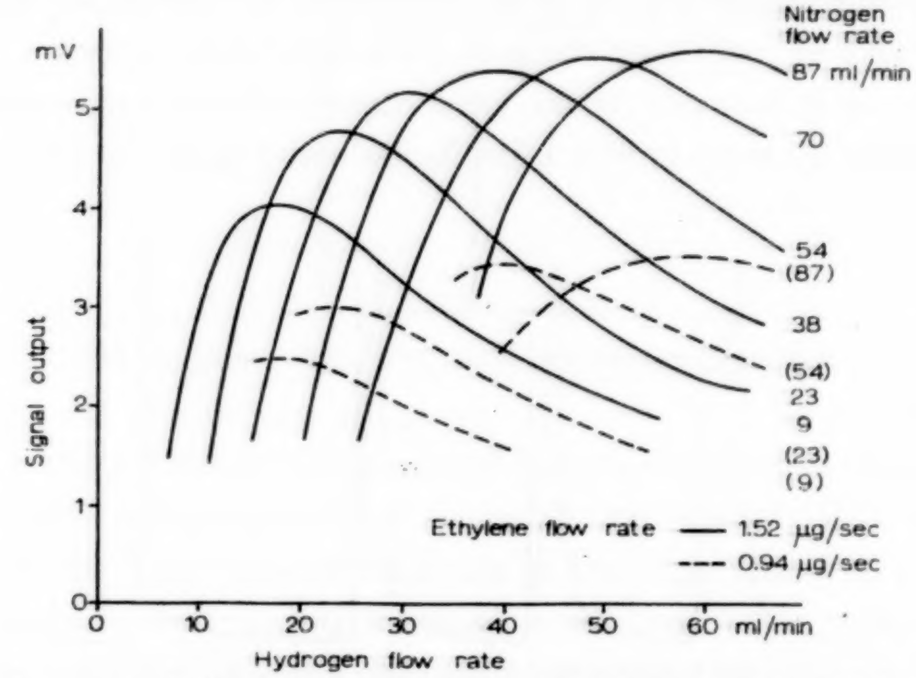

Fig. 4. Effect of hydrogen and nitrogen flow rates on ion formation, (conditions as for Fig. 3 with probe–jet distance 5 mm).

According to the response curves given here and the background noise limit given by KIESELBACH[4], the sensitivity limit of the detector is better than 3.10^{-7} µg/sec, and the response therefore covers a range of over 10^8 to 1 up to 5 % v/v at the detector (10 % in the nitrogen carrier gas stream). The results of this investigation and that reported by DESTY, GEACH AND GOLDUP[3] show strict linearity over the range from 50 µg/sec down to 3.10^{-4} µg/sec, a ratio of over 10^5 to 1.

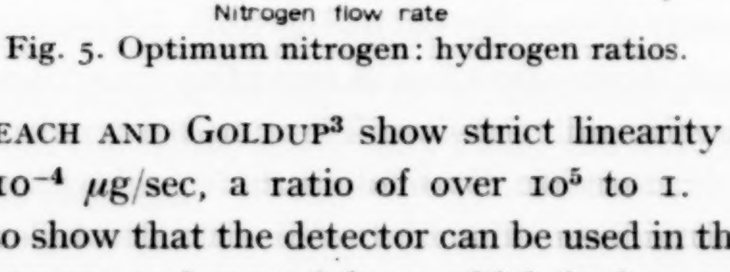

Fig. 5. Optimum nitrogen: hydrogen ratios.

The above data also show that the detector can be used in the medium sensitivity range for hydrogen, nitrogen and materials to which it does not normally respond. This is done by using a large hydrogen flow rate to give an initially high background signal[8] or by bleeding in a constant stream of, *e.g.*, coal gas and operating at a non-optimum nitrogen : hydrogen ratio.

Abnormal non-linearity effects

It is generally considered that the flame ionisation detector is relatively insensitive to inorganic gases, including carbon disulphide. However, in the latter case it has been found that an initial sensitive response is obtained which is quickly suppressed at higher concentrations, resulting in peak inversion as shown in Fig. 6, which was obtained with a 0.02 in. capillary column. This behaviour appears to be peculiar to carbon disulphide as it has not so far been observed with any other material using the present electrode system. It was initially believed that the effect was due simply to inhibition of ion formation by the products of combustion. However, addition of sulphur dioxide (or hydrogen sulphide) to coal gas which was then fed directly to the detector did not inhibit the signal due to the coal gas alone.

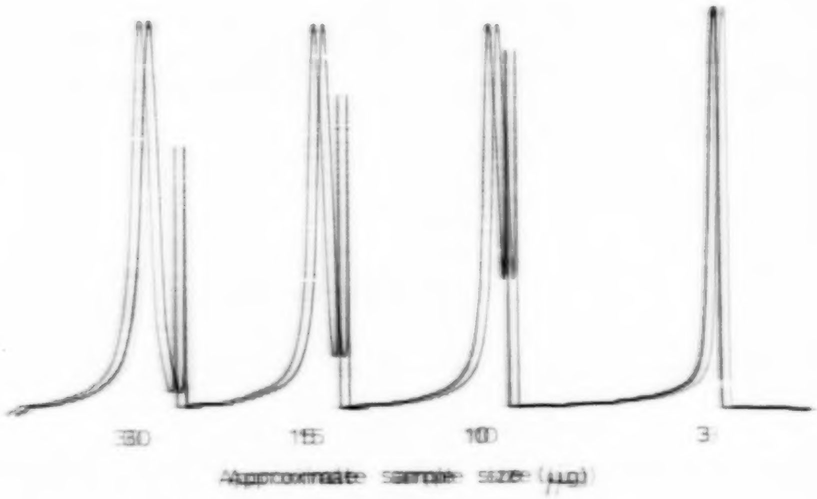

Fig. 6. Response of carbon disulphide.

Novák and Janák[13] have reported peak inversion effects with hydrocarbons at concentrations of about 8 µg/sec (0.065% by volume), which is to be compared with the peak concentration of carbon disulphide in this case of 0.4 µg/sec (0.03% by volume). However, they showed that their results were due to the design of their electrode system which would be expected to produce non-linear effects.

Use of non-linear response

Although a strictly linear detector response is desirable for many applications, the analysis of wide range samples may involve a large number of sensitivity changes. Range extension by automatic offset without change in sensitivity is unsatisfactory due to the greatly increased recorder balancing time. Extension of range by automatic sensitivity changes also causes some loss in recording speed, and the resulting chromatogram is untidy and difficult to follow. This can be avoided by the use of a logarithmic or other non-linear scale. By feeding the signal to the recorder via a suitably loaded multitap potentiometer geared to the recorder balancing motor an almost true logarithmic response can be obtained[19]. An alternative quasi-logarithmic response system is described below which is applicable to any high-impedance ionisation detector.

Under normal conditions of operation of the flame ionisation detector the current flowing round the circuit shown in Fig. 7 is determined by the effective flame resisttance, R, and the battery voltage, V, the grid input resistor, r, being much less than R. If, however, the grid input resistor is comparable with the flame resistance, the voltage drop across r is given by $v = rV/(r+R)$. From this function the relation between v/V and r/R is plotted on a semi-logarithmic scale in Fig. 8. For constant V and r, the signal output over the middle of the range shown in Fig. 8 is almost linearly proportional to log (r/R) which is proportional to log (flame conductivity) and hence to log (sample concentration). The response is linear in the high sensitivity region, logarithmic in the intermediate region, and tends to a limiting value at large sample sizes. It should be noted that the baseline reading represents the total background current in the flame circuit. In practice the low-sensitivity end of the response curve will differ somewhat from that shown in Fig. 8 since no allowance has been made for the variation of resistance with applied voltage[10] of the high-value resistor, r, nor for the reduction in voltage across the flame which may affect the ion collection efficiency as discussed in the previous sections.

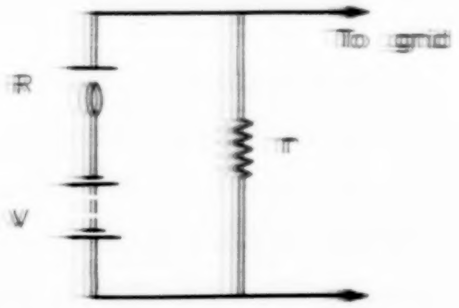

Fig. 7. Basic circuit of flame ionisation detector.

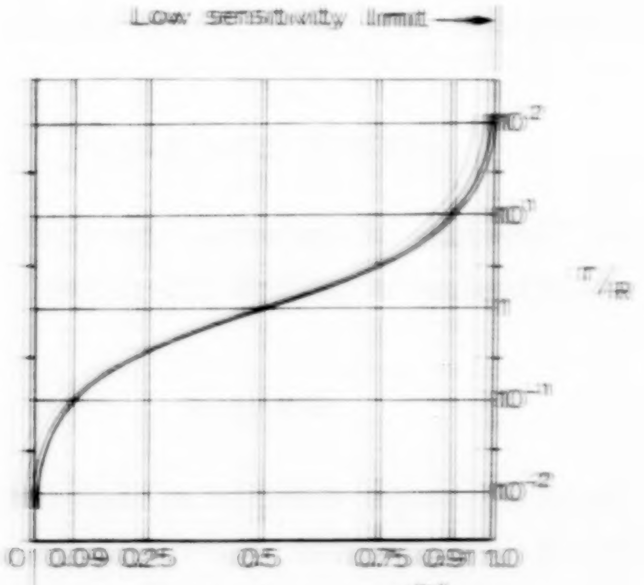

Fig. 8. Non-linear response curve.

Mechanism of ion formation

The abnormally high degree of ionisation in a hydrocarbon flame cannot be simply accounted for on the basis of the thermal ionisation of hydrocarbon molecules (which have ionisation potentials in the region of 9–12 eV). A possible mechanism of ion formation based on the formation of carbon aggregates and their subsequent thermal ionisation has been suggested[11], and a recent investigation of the flame ionisation detector has been regarded as supporting this view since the number of molecules required to produce one ion is roughly equal to the number of carbon atoms required to form suitably sized aggregates[2]. An alternative and more likely explanation, however, is possible.

It is supposed that due to the reactions occurring in the flame an intermediate "reaction complex" is formed with one of the carbon atoms in the molecule. The complex so formed will exist in an entirely different (excited) electronic and vibrational state from that of the parent hydrocarbon. Subsequent thermal ionisation of this complex may therefore occur with a relatively high probability since the limitation imposed by the normally high ionisation potential of the parent molecule is removed. A linear detector response can be expected from such a mechanism, and the proportionality of the response per mole to the number of carbon atoms in the molecule[1-3,5] is explained by the proportionately larger number of centres at which reaction may be initiated. Assuming further that carbon–oxygen attachment prior to formation of carbon dioxide is the critical step, the insensitivity of carbon monoxide and carbon dioxide is explained and also the lower sensitivity of oxygen-containing molecules compared with the corresponding hydrocarbons.

Formation of (hydrated) hydrogen ions as part of the basic ion formation mechanism, suggested by mass spectrometric studies[12], appears to be unlikely due to the sensitive response obtained with carbon tetrachloride.

ACKNOWLEDGEMENTS

The author thanks the Directors of Imperial Chemical Industries of Australia and New Zealand Ltd. for permission to publish this paper.

SUMMARY

Non-linearity of the flame ionisation detector at high concentrations has been shown to be caused by inefficient ion collection, due to ion recombination and space charge effects, rather than a basic non-linearity of ion formation. This can be overcome by using a flat probe electrode which subtends a large angle at the jet or a hat-shaped collector in conjunction with an adequate probe–jet voltage. With a detector unit similar to that described previously[5,6] a linear response up to 10% sample in the nitrogen carrier gas stream (5% at the jet) has been obtained (50 μg/sec at 25 ml/min nitrogen). A non-linear response system to avoid sensitivity changes with wide range samples is described. A possible explanation of the abnormally high degree of ionisation found in hydrocarbon flames is suggested.

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GAS CHROMATOGRAPHY OF SOME BROMINATED METHYL OCTADECANOATES[*]

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(Received November 7th, 1960)

The identifications of 12-bromo- and several dibromooctadecanoates by gas chromatography have been investigated. On a polyester column, any polar effects of the secondary bromine substituent are of a lower order of magnitude than the effect of weight alone. The fact that the data obtained from the bromo compounds fall very close to an extrapolated plot of molecular weight *versus* log of the retention time for saturated methyl esters implies that the principal effect of the bromine groups is a contribution to the molecular weight. The relative retention times of methyl 12-bromooctadecanoate, synthesis of which is reported here, and methyl *threo*-9,10-dibromooctadecanoate obtained from oleic acid are presented in Table I. It is obvious that with a retention time more than twenty times that of stearate, the dibromo compounds will not interfere with analyses of bromine-free saturated accompaniments. The tentative characterization of methyl *threo*-9,10-dibromooctadecanoate as having a retention volume of 1.18 with respect to methyl stearate[1] appears in light of the data presented here to be in error.

TABLE I

EFFECT OF BROMINE SUBSTITUENTS ON RETENTION TIME OF METHYL OCTADECANOATES

Compound	*Relative retention time*	
Methyl *threo*-9,10-dibromooctadecanoate	21[*]	22[**]
Methyl 12-bromooctadecanoate	5.7	6.1
Methyl arachidate	2.0	1.9
Methyl stearate	1	1
Methyl palmitate	0.54	0.53

[*] 3 ft. column 183°, flash heater 233°, detector cell 223°, argon flow 150 ml/min.
[**] 6 ft. column 186°, flash heater 235°, detector cell 239°, argon flow 90 ml/min.

The polyester packing was found to be insensitive to the differences between *erythro* positional isomers. Methyl *erythro*-9,10-dibromooctadecanoate from elaidic acid and the *erythro*-11,12-dibromo isomer from vaccenic acid[**] had the same elution time.

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[*] This work was supported by Research Grant H-4120 from the United States Public Health Service.

[**] Fluka AG, Chemische Fabrik, Switzerland.

A mixture of *erythro*-11,12- and *erythro*-12,13-dibromooctadecanoates obtained from "vaccinic" acid* had the same retention time as the other *erythro* isomers without any apparent peak broadening due to the heterogeneity of the eluate.

Contrary to the indifference of the polyester stationary phase to positional isomerism of these *erythro* vicinal dibromides, a difference was observed between the two 9,10-dibromooctadecanoate diastereoisomers, Table III. The *erythro* compound was eluted more rapidly from the column than the *threo* isomer, but the 5% difference in their retention times is apparently insufficient to permit their complete separation in such columns.

TABLE III

RETENTION TIMES OF DIASTEREOISOMERIC METHYL DIBROMOOCTADECANOATES

Compound	*Retention time*	
Methyl *erythro*-9,10-dibromooctadecanoate	48.8 min*	204 min**
	48.5	
Methyl *threo*-9,10-dibromooctadecanoate	51.0	215
	51.6	
Methyl *erythro*-11,12-dibromooctadecanoate	48.3	
Methyl *erythro*-11,12- and -12,13-dibromooctadecanoate (equal mixture)	48.3	
Methyl stearate	2.4	8.6

* 3 ft. column 183°, flash heater 233°, detector cell 223°, argon flow 150 c.c./min.
** 6 ft. column 185°, flash heater 218°, detector cell 242°, argon flow 192 c.c./min.

In a consideration of the difference observed between diastereoisomers, a useful qualitative rule may be formulated on the basis of a postulated relationship between dipole moment and retention times of isomers on a polar column. The *threo* isomer, being less symmetrical than the *erythro*, will have a higher dipole moment. Increased dipole–dipole interaction between the solute (the fatty ester) and the solvent (the polyester liquid phase of the column) shifts the partitioning of the solute between the gas and liquid phases to favor solubility of the *threo* over the *erythro* isomer in the liquid phase and thus causes a longer retention time for the *threo* compound.

A generalization of this kind is not meant to deny the contribution of other factors affecting the approach to thermodynamic equilibria which determine the retention times of various solutes. Rather, in this case, there is provided an opportunity to dissect dipole effects from other parameters affecting the partitioning potentialities of the system. The differential behavior of *cis* and *trans* isomers of unsaturated fatty esters on columns of this type may also be rationalized by recourse to this generalization.

The vapor phase separation of diastereoisomers in general has been little exploited, although references may be made in this regard to a report of separation by conventional distillation[2] and to another involving activated charcoal as the non-volatile component in a gas-phase separation[3]. Gas–liquid chromatography has been successfully employed in separating *meso* and racemic 2,3-dichlorobutane[4] and diastereoisomeric 2,3,4-trimethylhexanes[5].

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* Nutritional Biochemicals Corp., Cleveland, Ohio. This sample was shown to be an approximately equal mixture of 11- and 12-octadecenoic acids (personal communication from Dr. ARMAND FULCO).

To dispel any concern over the possibility of decomposition having occurred during these chromatographic studies, samples of methyl 12-bromo- and of *threo*-9,10-dibromooctadecanoate were recovered from the column and rechromatographed. No evidence of decomposition was discernible. Further evidence of the stability of these dibromides under the operating conditions employed here was obtained by debrominating (with zinc in methanol) a recovered sample of chromatographed *threo*-9,10-dibromide and identifying the product as methyl oleate on the basis of its gas-phase chromatographic behavior.

EXPERIMENTAL

The chromatographic runs were made using a Barber Coleman Model 10 Chromatograph using argon and a radium detector. The glass columns contained 20% succinic acid–ethylene glycol polymer on siliconized Johns-Manville Chromosorb, 80–100 mesh. The samples were injected as 1% acetone solutions with the exception of the samples which were to be collected, and these were injected neat. The operating conditions are given in the tables. Retention times were measured from solvent front to the maximum height of peak.

threo-9,10-Dibromooctadecanoic acid was prepared according to the method of NEVENZEL AND HOWTON[6].

erythro-11,12-Dibromooctadecanoic acid was prepared from vaccenic acid by adding the calculated amount of Br_2 in CCl_4 to a CCl_4 solution of the acid. "Vaccinic" acid was brominated in a similar manner to yield a mixture of *erythro-10,11* and *11,12-dibromooctadecanoic acid*. ***erythro-9,10-Dibromooctadecanoic acid*** was prepared similarly from elaidic acid. All of the acids were esterified with diazomethane prior to chromatography. A combination of gas chromatography and infrared spectrophotometry showed the vaccenic and "vaccinic" acids to be at least 75% *trans*.

12-Bromooctadecanoic acid. Since there is no published report of this compound, its preparation will be given in detail. A sealed Pyrex tube containing 6.0 g (0.02 mole) of 12-hydroxyoctadecanoic acid (Eastman) and 25 ml of 25% HBr in acetic acid (w/w) was heated at 100° for 16 h. The dark brown oil remaining after removal of the solvent and excess HBr was taken up in 30–60° petroleum ether, washed and dried over $MgSO_4$. Two crystallizations from 30–60° petroleum ether at −20° gave 5.3 g of colorless crystalline material melting at 33–35°. A small quantity was crystallized 3 additional times to raise the melting point to 37.0–37.8°.

Anal.: Calcd. for $C_{18}H_{35}BrO_2$: C, 59.49; H, 9.71.
Found: C, 59.66; H, 9.67.

SUMMARY

During gas chromatography on a polyester column, the principal effect of bromine substituents on a fatty ester molecule was to increase the retention time in proportion to the increase in molecular weight. Secondary effects arose from configurational isomerism. Methyl *threo*-9,10-dibromooctadecanoate was retained longer than the *erythro* diastereoisomer. The chromatographic difference between diastereoisomers was attributed to a change in dipole moment.

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A COMBUSTION METHOD FOR THE QUANTITATIVE ESTIMATION OF CHROMATOGRAPHIC SPOTS

I. DETERMINATION OF ORGANIC COMPOUNDS CONTAINING FLUORINE

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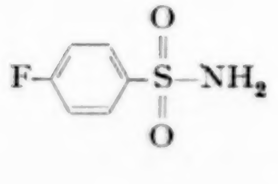
(Received November 25th, 1960)

GENERAL INTRODUCTION

The quantitative determination of organic compounds that have been separated by paper chromatography or electrophoresis is generally carried out by means of a colour reaction. The colour intensity is usually measured after elution, but it can also be determined directly on the paper, the absorbance being measured under U.V. light. Most colour reactions are based on a reaction with a functional group. Few or no quantitative determinations of spots are, however, based on the determination of a single element present in the compound under investigation.

In the procedure published in 1955 by SCHÖNIGER[1] for the micro-determination of elements in organic compounds, a method has been designed that is very suitable for the quantitative determination of spots separated by chromatography or electrophoresis.

In the so-called SCHÖNIGER combustion, an organic compound is burned in an erlenmeyer flask containing oxygen and the combustion gases are absorbed in an appropriate liquid. In this way the organically bound elements are transformed quantitatively into the ion form. The combustion of for instance *p*-fluorosulphonamide with water, using peroxide as absorption liquid, can be summarised as follows:



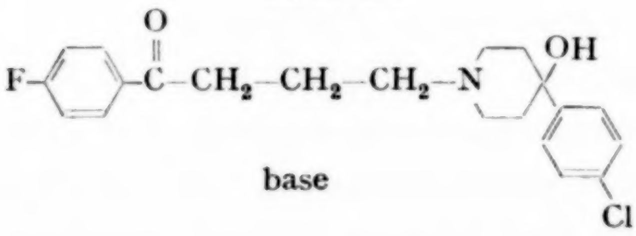
$$\begin{array}{l} C \longrightarrow CO_2 \\ H \longrightarrow H_2O \\ N \longrightarrow N_2 \text{ and a small amount of } NO_3^- \\ S \longrightarrow SO_4^{2-} \\ F \longrightarrow F^- \end{array}$$

The numerous publications and review articles[2-5] that have appeared since this method was first published, provide evidence of the great progress it has made in organic micro-chemistry.

For a description of the (very inexpensive) apparatus and the procedure, the reader is referred to the original paper[1]. In this procedure an aliquot of the substance is weighed so as to obtain ± 0.05 mequiv. of the element to be determined. This amount is, however, much larger than those obtained by paper chromatography or electrophoresis, as the spots often contain not more than 0.1 μequiv. of the element to be determined. The existing micro-methods for the determination of the following organically bound elements in the oxygen flask, C, Cl, Br, I, F, S, P, As, B, Zn, Hg, Cd and Se[6], should therefore be modified and scaled downward (sub-micro scale), or new methods should be designed.

SUB-MICRO DETERMINATION OF ORGANICALLY BOUND FLUORINE

Among many butyrophenone derivatives synthetized in this laboratory, a new compound has been prepared with potent neuroleptic properties and bearing some resemblance to the phenothiazine derivatives. This compound, called Haloperidol, is highly active in very small concentrations[7].

Structure	Laboratory name
 base	R 1625

The numerous experiments, the main object of which was to gain a better knowledge of the mode of action of the drug, were also made with the aim of studying the *in vivo* and *in vitro* metabolism of Haloperidol in rats.

Various reactions were performed to characterise the compound after extraction from biological material. A variety of ketone reagents[8–10] were used, all of which gave negative results. This may perhaps be attributed to an interaction between the negative carbonyl group and the positive nitrogen atom in the heterocyclic nucleus. Reactions for the heterocyclic nitrogen atom proved to be qualitative but not quantitative[11]. The possibility was, therefore, considered of carrying out a chlorine or fluorine determination after chromatographic separation of the compound. Since animal tissues contain much more chlorine than fluorine, a fluorine determination will be more specific. Furthermore, no basic organic fluorine compounds (which would also be extracted in the method used) seem to be present in rat tissue, urine or faeces, while the inorganic salts containing fluorine, such as CaF_2 and NaF are not determined. This is important, in view of the fact that the dose of Haloperidol under investigation is only 40 γ per rat, which corresponds to 2 γ F/rat. If NaF and CaF_2 were also determined, the blank value would be much too high.

The method used, which is described below, can be summarised as follows:

1. Extraction of the substance from biological material.
2. Concentration of the extract.
3. Chromatography.
4. Visualisation of the spots.
5. Determination of the fluorine content of the spots.

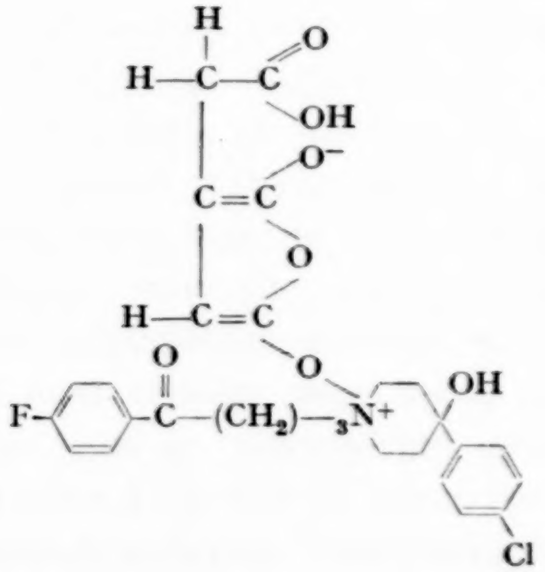
Methods

Extraction from biological material (urine, faeces, tissue). The sample is adjusted to pH 12 with 10 *N* NaOH and shaken with 5 vol. of ethyl ether. The ether layer is evaporated to ± 5 ml *in vacuo* and the base extracted with 2 vol. (10 ml) 0.1 *N* HCl.

Concentration of the extract. After extraction of a substance, either in water or in organic solvent, the total volume can be reduced to a very small known amount (*e.g.* from 10 ml to 50 μl) by the very interesting method described by CLARKE AND HAWKINS[12]. This method allows the determination of the total amount of extracted substance on a single chromatogram.

Chromatography. Experiments were made with buffered paper and the solvent described by CURRY AND POWELL[11]. The R_F value in this solvent (the upper layer of 50 ml butanol, 50 ml water, 1 g citric acid) was, however, between 0.95 and 1.0. The buffer paper was retained, but another solvent was used: methanol–amyl alcohol–benzene–water, 40:20:40:5. To avoid interference of the fluorine determination, the solvents should be free of traces of heavy metals (Dithizon test).

Visualisation of the spots. The spots are visualised under an U.V. lamp. It is, however, possible to spray with a reagent containing elements that after combustion do not interfere with the fluorine determination. A modified DRAGENDORFF reagent[13] containing bismuth cannot be used because bismuth interferes with the fluorine determination; a reaction with, for example, *cis*-aconitic acid in acetic anhydride[14,15] is, however, possible. In this reaction the following violet coloured complex is probably formed:



Buffered paper may not be used in this case, as alkali salts of organic acids give a positive reaction[15]. This reaction will be studied further; a sensitivity of 0.1 γ Haloperidol was obtained in a preliminary investigation.

Determination of fluorine. The decrease in fluorescence of the aluminium–morin complex induced by increased quantities of fluoride ion forms the basis of a standard method for fluoride determinations. This procedure was used by BOUMAN[16] for the determination of fluorine in the range of 0.005 γ – 0.08 γ. We have found, however, that the complex formed is very unstable and that the measurements vary frequently.

When 25 γ of fluorine are present after chromatography, a titration can be performed in a volume of 5–10 ml with a relative error of ± 2%. This method is a modification of a micro-titration[5] in which thorium nitrate is used as the titrant and alizarinsulphonate–methylene blue as the indicator.

For smaller amounts (0.1–2 γ F) a slightly modified method of SINGER AND ARMSTRONG[17] was found satisfactory: the decoloration of the zirconium–Eriochrome Cyanine R complex is proportional to the fluoride concentration.

Apparatus

A modified SCHÖNIGER Erlenmeyer flask of 150 ml with a B 24 stopper with platinum wire and a small valve fitted at the bottom of the Erlenmeyer, as shown in Fig. 1.

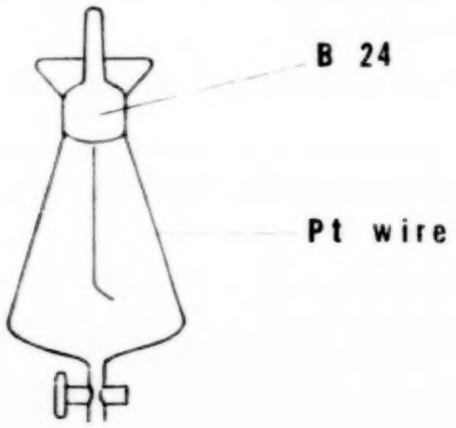


Fig. 1. Modified SCHÖNIGER oxygen flask.

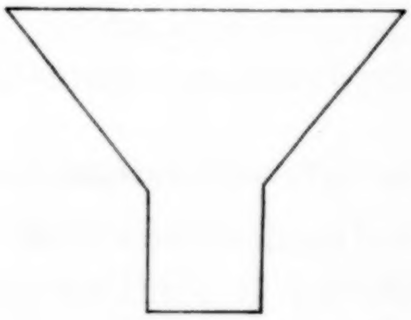


Fig. 2. Submicro titration vessel. Full size. The liquid in the upper part must be evaporated before the titration is carried out in the cylindrical part.

Colorimeter tubes, size 10 ml (borosilicate glass).

Titration vessel for submicro titration as shown in Fig. 2.

Microburette: 0.3 ml (Prolabo).

Apparatus for drying strips of filter paper (Fig. 3).

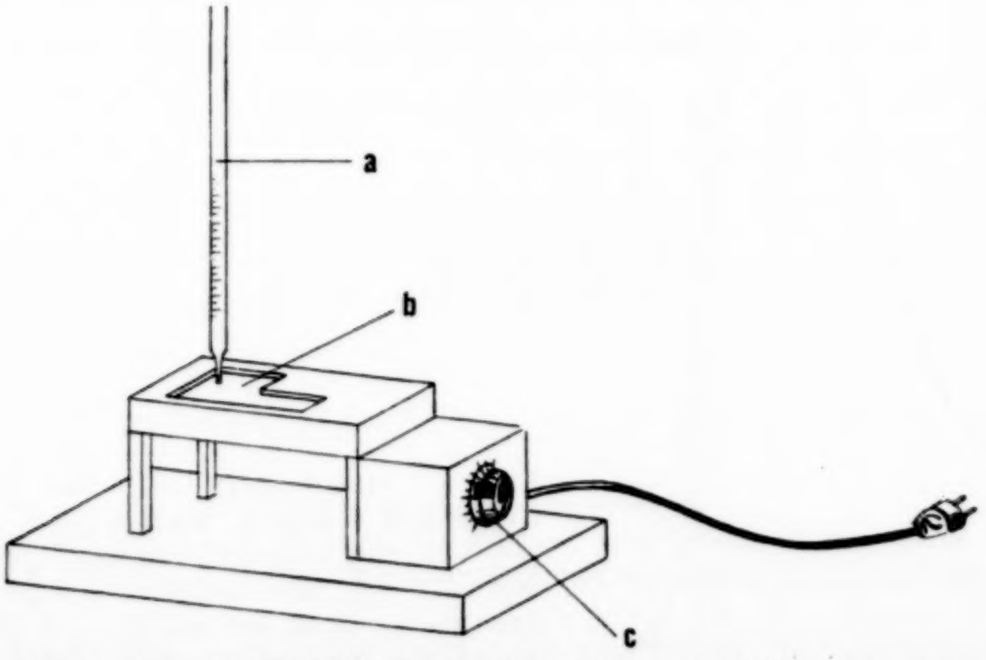


Fig. 3. Apparatus for drying strips of filter paper. (a) Pipette of the apparatus of CLARKE AND HAWKINS[12]; (b) a spot cut out from a chromatogram; (c) variable resistance.

Eppendorf-Colorimeter with mercury-lamp, 546 mμ filter and cuvettes of 4 cm light path.

Reagents

Redistilled water: Laboratory distilled water is deionized by passing it through a mixed-bed resin (Elgastat) until the resistance reaches 800,000 Ω/cm. The water is stored in a polyethylene bottle.

Solution a: 210.7 mg of Eriochrome Cyanine R (Merck) dissolved and diluted to 100 ml with water and stored in a polyethylene bottle.

Solution b: 30.5 mg zirconylchloride octahydrate (Merck) dissolved in 161.4 ml concentrated hydrochloric acid and diluted to 200 ml with water (polyethylene bottle).

Reagent solution: 1 vol. of solution a added to 2 vol. of solution b. This solution is prepared fresh daily.

Fluoride stock solution: 22.1 mg p.a. sodium fluoride (Merck) dissolved in 100 ml of water (polyethylene bottle).

Standard solution: 2 ml of the stock solution diluted to 200 ml with water (1 γ F/ml).

Calibration curve with sodium fluoride

In a series of 6 colorimeter tubes (10 ml), 0, 0.4, 0.8, 1.2, 1.6 and 2.0 ml of the standard fluoride solution (1 γ/ml) are placed; 0.5 ml of the reagent is added and the solution is

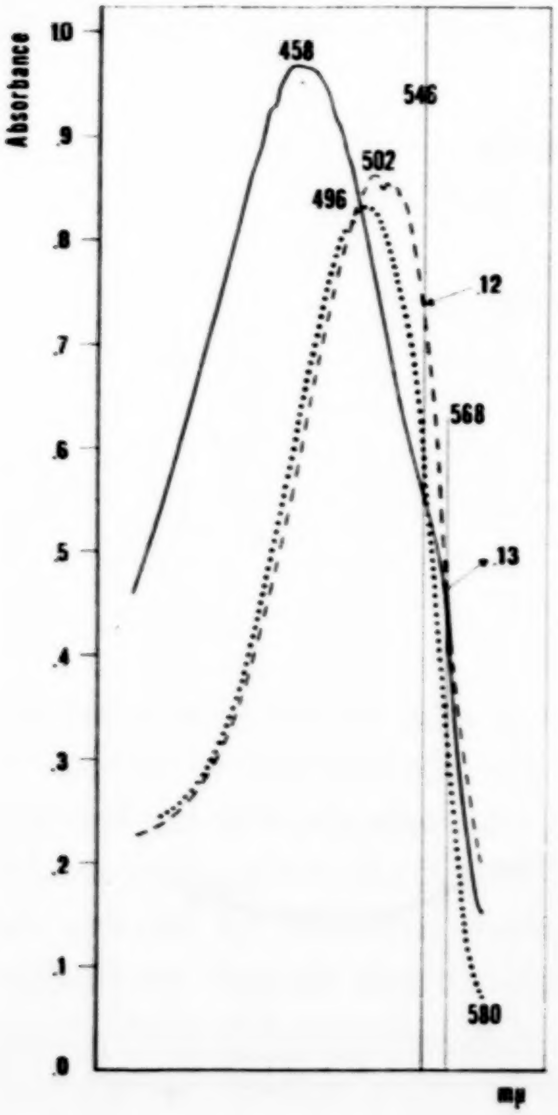


Fig. 4. Absorbances of: the Eriochrome Cyanine R solution, with a peak at 458 mμ (——); the reagent complex, peak at 502 mμ (– – – –); the reagent complex + 2 γ fluoride, peak at 496 mμ (. . . .). The absorbances are measured *versus* water in a Beckman DK$_2$ apparatus with cuvettes of 1 cm.

then diluted to 10 ml with water. The tubes are shaken and the extinctions measured 1 h later. The solutions containing fluoride are used as blank solutions. The extinctions are measured at 546 mμ in a cuvette of 4 cm. Fig. 4 was drawn in order to find a wavelength at which there is sufficient difference between the blank and the sample.

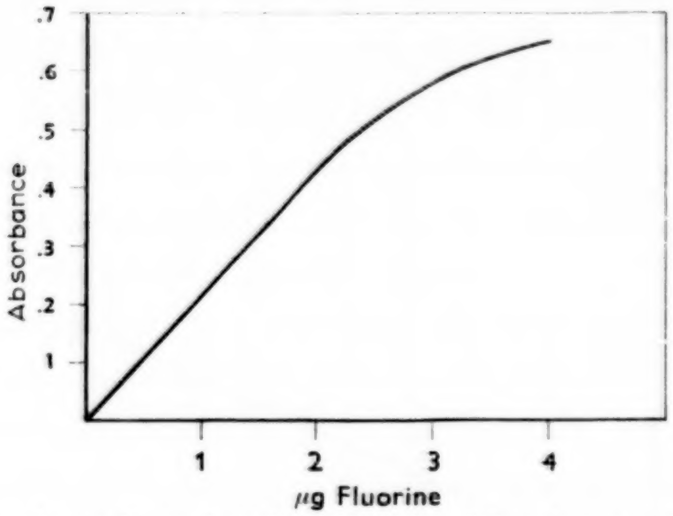


Fig. 5. Calibration curve of a sodium fluoride solution. Absorbances of the zirconium–Eriochrome Cyanine R complex with 0–2 γ fluoride in a total volume of 10 ml.

As can be seen in Fig. 4, the region between 580 mμ and 540 mμ is very suitable for measurements. SINGER AND ARMSTRONG made the measurements at 568 mμ in a Coleman spectrophotometer. In our method, the measurements are performed at 546 mμ. Both measurements are almost equivalent.

Fig. 5 shows that the relation between fluoride concentration and extinction is linear within the range of 0–2 γ F.

Calibration curve after combustion of an organic substance containing fluorine

A solution containing 1 mg Haloperidol "purissimum" per ml of 0.1 *M* tartaric acid is introduced into a microburette (0.3 ml); 0, 8, 16, 24, 32 and 40 μl of this solution are then placed on buffered paper. The spots are cut out under an U.V. lamp as indicated in Fig. 6. The strips are fixed to the platinum wire of the B 24 stopper (Fig. 1).

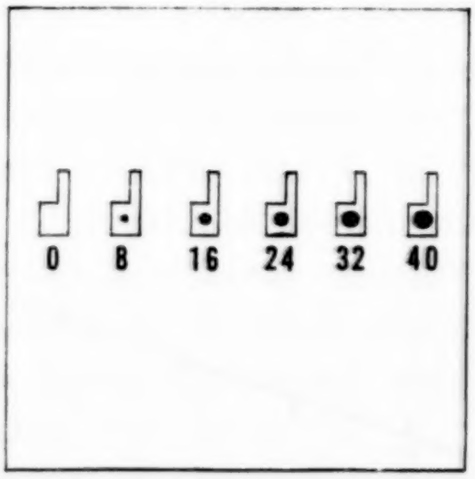


Fig. 6. Six spots are placed on a Whatman No. 1 paper and dried; their outlines are traced with a pencil under U.V. light. The spots are cut out along the pencil line.

The combustion flask (Fig. 1) is filled with 2 ml water and flushed with a strong flow of oxygen for 30 sec; the strip is ignited and the flask closed. After absorption of the combustion gases, the neck around the stopper of the combustion flask and the platinum wire are rinsed with 2 ml water. The valve is opened and the absorption liquid collected in a colorimeter tube. The flask is again rinsed with 5 ml water and this is added to the first 4 ml, 0.5 ml reagent is added, the total volume diluted to 10 ml and the extinction measured after 1 h as described above for the NaF solution. Fig. 7 indicates that after combustion the same extinctions as in Fig. 5 are found for the same amounts of fluorine. The deviations are however 20–30 %.

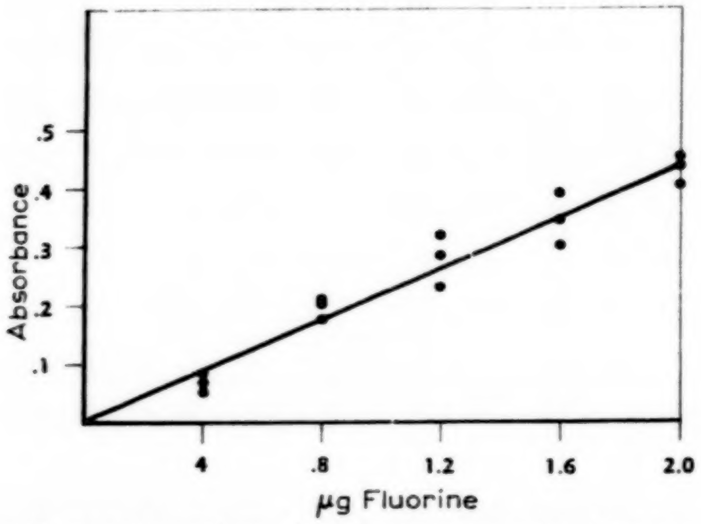


Fig. 7. Calibration curve after combustion of various amounts of an organic substance (Haloperidol) containing 0–2 γ fluorine. Absorbances of the zirconium–Eriochrome Cyanine R complex.

Calibration curve after chromatography

The calibration curve was drawn using the same method as for Fig. 7, except that the 6 spots were placed at one end of the paper. After ascending chromatography the papers are dried at 65° for 30 min. The spots with R_F values between 0.75 and 0.80 that are visible under an U.V. lamp are cut out. The procedure is further carried out as described for the calibration curve of Fig. 7.

RESULTS

From Fig. 8 it can be seen that after chromatography the extinctions for the same amounts of fluorine have decreased, probably owing to loss during the procedure.

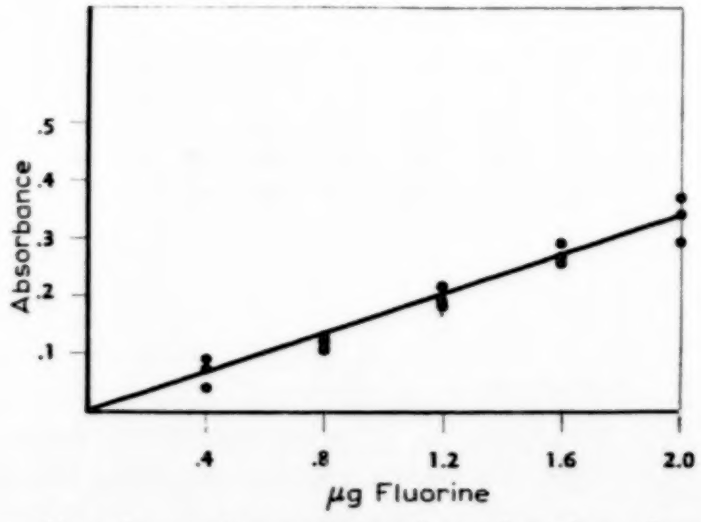


Fig. 8. Calibration curve after chromatography and combustion of various amounts of an organic substance (Haloperidol) containing 0–2 γ fluorine. Absorbances of the zirconium–Eriochrome Cyanine R complex.

The average values of the results, calculated on the "best fitting line", varied between 85 % and 110 %.

DISCUSSION

When the test solution contains only one fluorine compound, it is not necessary to separate the compound by chromatography; the compound to be determined can be placed on the strip after evaporation of the solvent. The extract is then introduced into the pipette described by CLARKE[12] and dripped onto the strip which is placed on an adjustable heating disk (Fig. 3). A volatile solvent (alcohol, ether) is preferable because the evaporation is more rapid.

Aliquots of Haloperidol up to 1 mg (50 γ F) were added to 50 ml rat urine and part of the ether extract was added dropwise onto the strip. The recovery was 90 %.

SINGER AND ARMSTRONG[17] studied the influence of sulphate and phosphate on the fluorine determination and observed that less than 30 γ sulphate did not interfere and that 5 γ phosphate gave the same extinctions as 0.1 γ fluorine. The new method of determining fluorine described by BELCHER *et al.*[18, 19] (a direct colorimetric determination) might give better results. This will be investigated in the future.

The method described above is not limited to fluorine. Determinations of sulphur-containing amino acids should be possible by means of a submicro-sulphate determination and phosphorus in a compound by means of a phosphate determination. This will also be investigated in the future. In brief, any substance containing an element that can be determined with the oxygen flask lends itself to this technique. Carbon determinations can be made by eluting the substance in a suitable solvent, transferring it dropwise to a combustion boat and igniting it electrically. A combustion flask similar to those already described[20–22], but containing a valve at the bottom of the flask, should be designed.

Substances separated on chromatoplates can be determined as follows: the spot with the layer is cut out and placed in a centrifuge tube, an appropriate solvent is added and the solution centrifuged. The supernatant is pipetted onto a strip (Fig. 3).

Compounds containing no characteristic SCHÖNIGER element (*i.e.* compounds containing only C, H, N and O) can be determined by allowing them to react with a compound which does have a characteristic element and then making a chromatogram of the complex. Adrenaline and noradrenaline (C, H, N, O) form stable boric acid complexes[23]. The percentage of boron can be determined after chromatographic separation by a recently described fluorimetric method[24].

Preference should be given to heavy metal complexes providing they do not react with the platinum wire during combustion, because there are very accurate methods for such determinations.

Much depends on the accuracy with which the element can be determined on the submicro-scale. If the accuracy is greater than that of a given colour reaction for the spot or if this reaction is not sensitive enough, it is certainly worth while trying the above described method for the quantitative estimation of spots separated by paper chromatography or by paper electrophoresis.

ACKNOWLEDGEMENTS

We are indebted to Mr. RENÉ DE MEYER for his excellent technical assistance and helpful suggestions, and to Mr. VAN OFFELEN for drawing the figures. It is also a pleasure to thank Miss ANNIE CLAESSENS and Miss SCHUERMANS for the typescript and the help in the translation.

SUMMARY

A method for the quantitative estimation of chromatographic spots of fluorine-containing organic compounds has been developed. The procedure is based upon the colorimetric determination of the fluoride ion (range 0–2 γ F) formed after combustion of the fluorine-containing spot in a "SCHÖNIGER oxygen flask". The possibility of using this method for the determination of other elements is discussed.

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STANDARD IONOPHORETIC MOBILITIES OF VARIOUS BIOCHEMICALS, IN AMARANTH UNITS, AT SEVERAL pH VALUES FROM 3.3 to 9.3

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(Received November 8th, 1960)

The method of paper ionophoresis described by WERUM *et al.*[1] introduced three dyes as reference standards for the measurement of the mobility of charged compounds. The mobility scale proposed was the distance between spots of the uncharged dye, Apolon, and the negatively charged dye, Amaranth, which was defined as 100 *Am* units. The initial report was limited to a detailed presentation of the technique, with only a few examples of its application to the characterization of organic compounds.

The purpose of this second report is to present more data on the *Am* values of charged compounds of biochemical interest. The method used was exactly that described by WERUM *et al.*[1]. No comprehensive survey of all known biochemicals was intended, since the method will surely be considerably improved by our own and other laboratories during the next few years.

EXPERIMENTAL

Methods used for detection of spots

Ionograms were usually extracted with acetone to remove excess buffer; for ionograms run with the pH 9.3 borate buffer (pH 9.3 B), a solution of 5% pyridine in acetone was used. For detection of amines and amino acids, 0.1% ninhydrin in acetone was satisfactory. For most carbohydrates, the periodic–benzidine reaction gave the most sensitive detection[1]. For most of the acidic and basic compounds, the bromocresol purple indicator reagent ID-2 of GORDON AND HEWEL[2] gave fairly good spots. Organic phosphate esters were detectable by the ferric-*p,p'*-methylenebis-(N,N-dimethylaniline) color reaction of GORDON[3], which is a minor modification of the ferric-sulfosalicylic acid method of WADE AND MORGAN[4]. A few compounds, such as riboflavin, were detectable by ultraviolet fluorescence.

Preparation of dinitrophenyl derivatives

These derivatives were prepared from 1 ml or less of a 0.05 *M* solution of each amino compound, by adding one drop of a 1 M solution of dinitrofluorobenzene in methanol and a few drops of 20 % diethylamine in methanol. This is a minor modification of the method of SANGER[5]. After one hour, the reaction mixtures were ionophoresed, without purification.

RESULTS

Present limitation of the Amaranth value in characterization of charged compounds

The experience gained in the present survey has shown that the *Am* value has more limitations than were anticipated in the earlier work. Since a difference of 0.1 pH unit in the background buffer can cause a 10 % variation in the *Am* value of a substance whose pK is near the pH of the buffer, the buffer pH should be controlled to ± 0.01 pH unit. If the solution spotted has a relatively high buffering power and a different pH from that of the buffer, the local pH change may cause some streaking of the spots and shifting of the *Am* value.

The data now show that no simple empirical equation can accurately relate the *Am* value to the molecular weight, as suggested by WERUM *et al.*[1]. For example, the equation $800/\sqrt{M}$ would predict *Am* values (per electric charge) of 84 for oxalic acid, 70 for agmatine, and 84 for lactic acid, but the observed values are 69, 69 and 102. Calculated molecular weights may therefore be 20 % too high or too low. However, this equation may be very useful in approximating the molecular weight of an unknown.

In addition, the data indicate that pK values calculated from ionophoretic mobility data in 30 % formamide buffers of high ionic strength may differ very greatly from pK values in dilute aqueous solution. It has long been known that the pK of substances with closely interacting ionizing groups is markedly affected by ionic strength; *e.g.*, pK_2 for phosphate is 7.2 in very dilute solutions but falls to 6.8 in a 0.1 M phosphate buffer[6], while substances such as acetate show much smaller decline in pK with increasing ionic strength. Organic solvents can also exert large and unequal effects on pK values; *e.g.*, the pK of acetic acid rises from 4.7 in water to 7.1 in 90 % ethanol, while the pK_1 value of glycine only rises from 2.5 to 3.8 and its pK_2 value from 9.8 to 10.0. Similar pK-shifts occur in the 30 % formamide system; *e.g.*, calculation of pK values from *Am* values by the method of WERUM *et al.*[1] for oxalic acid gives an estimated pK_2 (for the more weakly acidic carboxyl group) of 2.7, which is much lower than the value of 4.3 observed in dilute water solutions. The interaction between the adjacent carboxyl groups in oxalic acid is therefore greatly intensified in 30 % formamide solution. On the other hand, the estimated pK_2 for adipic acid is 5.5, which agrees with the value observed in aqueous solution, presumably because the two carboxyl groups are so far apart that interaction is negligible.

One factor that should be given consideration is the change in pK with temperature. Some pK values can shift by 0.05 pH units for a 10° change in temperature, because ionization increases as temperature rises. Since the buffer temperature is above ambient when paper electrophoresis is run at levels of the order of 10 mW per cm², the pK both of the buffer ions and of the ionizing groups of compounds being analysed will be altered. It is therefore desirable to specify not only the voltage gradient but the wattage level at which a sample has been run.

Possibly one of the largest sources of error is the uneven drying of the ionograms after they have been removed from the apparatus. However, this error can be miminized by cutting off the ends of the ionograms which are immersed in the buffer solution, blotting and drying the ionograms immediately after ionophoresis is finished. The use of multiple dye spots also helps to minimize this error.

From the above discussion it can be seen that in the comparison of *Am* values of unknowns with listed standard values, emphasis should be placed on the pattern of mobility change with pH rather than on the precise *Am* values; a single *Am* value in only one buffer is rarely sufficient.

Mobility data for organic bases

The tables are organized to facilitate identification of unknown compounds. Table I lists organic bases which have only positive (or zero) charge in the pH range from 3.3 to 9.3. A few of these acquire a negative charge in the pH 9.3 borate buffer.

TABLE I

COMPOUNDS HAVING ONLY ZERO OR POSITIVE CHARGE IN NON-BORATE BUFFERS*
(In order of decreasing *Am* values at pH 3.3)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
Ethylenediamine	197				159		73	38
Methylamine	180				187			
N-Aminoethyl-piperazine	180			155	156		95	
Spermidine	178				147		97	58
3-Dimethylamino-*n*-propylamine	175				156	150	90	
Piperazine	175				111		82	53
Tetraethylenepentamine	164			148	144		74	
1,4-Diaminobutane	163				163		131	78
N-Hydroxyethyl-piperazine	153			83	82		50	
Cadaverine	160				149		137	
Pyrrolidine	140				140		131	
Agmatine	137				137		123	
Ethanolamine	134				135		93	53
Glycinamide	125				112	84	3	
Arcaine	124						124	
3-Amino-1-propanol	120				119		92	61
3-Amino-2-propanol	117				117		70	41
3-Hydroxypiperidine	105				102		78	63
2-Phenylethylamine	100				100		75	
Ornithine	90	74	71			71	31	7
Amphetamine	90				89			
Glycyl-lysine	89	67			55	43	5	
Lysine	88	79				67	55	7
Tyramine	84				84		61	
2-Amino-2-hydroxymethyl-1,3-propanediol	80				80		14	—31

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* For the preparation of the buffer see ref.[1].

(continued on p. 134)

TABLE I (*continued*)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
Lysyl-glycine	80		63		49	37	8	
Tryptamine	79				79		70	
Cytosine	75		13	4			5	
γ-Aminobutyric acid	74	53	13		2		0	—12
3-Hydroxytyramine	74				74			
Arginine	68		66		62		45	33
Epinephrin	66				67		35	11
D-Glucosamine	61				58	38	6	—55
D-Galactosamine	59				58	44	10	—49

This suggests that a borate-complexing polyol structure is present, but WERUM *et al.*[1] have shown that the borate buffer in 30% formamide is apparently more alkaline than the corresponding pH 9.3 non-borate buffer because of hydrogen-bond interactions. A decrease in the *Am* value in the borate buffer may therefore indicate the presence of an ionizing group with a p*K* near 10. The compounds are listed in order of decreasing *Am* value in the pH 3.3 buffer. If an unknown compound has an *Am* value of 135 at pH 3.3, reference to Table I suggests that it may be ethanolamine or agmatine. If the value at pH 9.3 is close to 123, the compound is not ethanolamine. Since *Am* values are not yet reliable to much better than 5%, positive identification as agmatine is not possible. However, the compound is either a strongly monobasic substance of low molecular weight, like pyrrolidine, or a strongly dibasic substance of higher molecular weight, like cadaverine or agmatine.

Mobility of carbohydrates and polyols

Table II lists compounds that have zero charge in the pH range from 3.3 to 9.3 but acquire a negative charge in the pH 9.3 borate buffer. Nearly all the reference com-

TABLE II

COMPOUNDS HAVING APPROXIMATELY ZERO CHARGE, EXCEPT IN BORATE BUFFER, WHERE THEY HAVE A NEGATIVE CHARGE

(In order of decreasing negative *Am* values)

Substance	*pH* 7.2	*pH* 9.3	*pH* 9.3B
Riboflavin	0	—6*	—69
Isoriboflavin	0	—5*	—67
D-Glucose		0	—64
D-Arabinose		0	—63
D-Fructose		0	—62
D-Xylose		0	—62
L-Xylose		0	—61
L-Sorbose		0	—60
D-Glucoheptose		0	—60

(*continued on p. 135*)

* In pH 9.3 acetate buffer there was no movement from point of application.

TABLE II (*continued*)

Substance	*pH* 7.2	*pH* 9.3	*pH* 9.3B
Dulcitol		0	−59
D-Tagatose		0	−58
D-Galactose		0	−56
L-Fucose		0	−56
Mannitol		0	−56
Arabitol		0	−55
Adonitol		0	−53
Sorbitol		0	−52
D-Lyxose		0	−52
D-Ribose		0	−52
D-Melibiose		0	−51
D-Mannose		0	−49
Erythritol		0	−47
Sedoheptulose		0	−44
D-Turanose		0	−44
L-Rhamnose		0	−39
Lactose		0	−35
Maltose		0	−28
Raffinose		0	−24
Cellobiose		0	−24
Sucrose		0	−16
Proline		0	− 8

pounds are polyols that form borate complexes, but proline is an example of compounds with ionizing groups of p*K* near 10 that also can acquire a negative charge in the 30 % formamide borate buffer.

Mobility data for organic acids (except phosphate esters)

Table III lists acids having only a negative (or zero) charge in the pH range from 3.3 to 9.3, in order of decreasing *Am* value in pH 7.2 buffer. Acids that are fully dissociated at pH 7.2 were usually not run at higher pH values. Nearly all these acids are "normal" in that mobility relative to that of Amaranth is not decreased in the 9.3 borate buffer. "Abnormal" acids show this decrease, *e.g.*, most of the phosphate esters listed in Table IV, and also the reference dye, Brilliant Blue.

Mobility data for phosphate esters

Table IV lists these in order of decreasing negative *Am* value in the pH 9.3 borate buffer. This order is nearly the same as that in the pH 9.3 non-borate buffer, although values for pentose and hexose phosphate esters are higher in the absence of borate. Comparison with the data of WADE AND MORGAN[4] is possible in the pH 3.3 buffer, although they used a butyric acid–sodium butyrate buffer without added formamide. WADE AND MORGAN used orthophosphate ion as a mobility standard, and in our pH 3.3 system this ion has a mobility of 100 *Am* units. The calculated *Am* values from the data of WADE AND MORGAN are 21 for 5′-adenylic acid, compared to 25 in Table IV, and 55 for uridylic acid, compared to 58 in Table IV. There are differences, however, such as the calculated value of 100 for fructose-1,6-diphosphate, compared

TABLE III

COMPOUNDS HAVING ONLY ZERO OR NEGATIVE CHARGE IN NON-BORATE BUFFERS
(EXCEPT PHOSPHATE ESTERS, LISTED IN TABLE IV)
(In order of decreasing negative *Am* values in pH 7.2 buffer)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
Oxalic acid	—124		—137		—138			
trans-Aconitic acid	—63		—112		—137			
α-Ketoglutaric acid	—95		—107		—136			
Malonic acid	—90		—117		—136			
cis-Aconitic acid	—63		—109		—133			
Tartaric acid	—75		—111		—130			
Itaconic acid	—32		—90		—128			
Succinic acid	—18		—79		—127			
L-Malic acid	—46		—97		—126			
Pyruvic acid	—122		—123		—124			
Maleic acid	—124		—120		—121			
Glutaric acid	—12		—73		—121			
Tricarballylic acid	—30		—82		—120			
Glycolic acid	—35		—103		—117			
Citric acid	—55		—92		—116			
Carbamyl-aspartic acid	—39		—88		—114		—112	
α-Methylglutaric acid	—13		—62		—112			
Adipic acid	—13		—63		—110			
β-Methylglutaconic acid	—25		—80		—109			
Lactic acid	—38		—84		—102			
5-Hydroxy-2,4-dichloro-phenoxy-acetic acid	—76		—102		—100			
Mucochloric acid	—28		—81		—96			
2-Pyrrolidone-carboxylic acid	—56	—88	—90		—90		—96	
Diglycolic acid	—64		—78		—88			
2,4-Dihydroxycinnamic acid	—21		—66		—86		—91	—100
Orotic acid	—87				—84	—86	—86	
Barbituric acid					—80	—81	—79	
2-Chlorophenoxy-acetic acid	—54		—84		—80			
2-Hydroxyphenyl-acetic acid	—8	—47	—59		—80		—80	—78
Cysteinesulfinic acid	—79		—77		—77		—92	
Aspartic acid	—21		—71		—76		—81	—82
4-Chloro-2-methyl-phenoxy-acetic acid	—46		—77		—74			
2,4-Dichlorophenoxy-acetic acid	—52		—75		—73			
Glutamic acid	—7		—60		—72		—77	—77
Shikimic acid	—13	—40	—54		—71		—69	—80
Endophthalic acid	—11		—52		—71			
2, 4, 5-Trichlorophenoxy-acetic acid			—72		—70			
Quinic acid	—33	—65	—65		—69		—71	—60
3,4-Dihydroxyphenyl-acetic acid	—1	—32	—48		—67		—71	—88
Uracil-5-carboxylic acid	—9				—67	—68	—71	
Indole-3-acetic acid	—2		—36		—66			
Kynurenic acid			—63	—66	—65	—67	—66	
Uric acid			—23	—39	—63		—63	
2,4-Dichlorophenoxy-butyric acid					—63			
Xanthurenic acid	—60		—60	—57	—62	—61	—61	

(*continued on p. 137*)

TABLE III (*continued*)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
2-Hydroxycinnamic acid	—4		—41		—55		—65	—68
Gibberellic acid	—10		—42		—54			
Xanthosine				—28	—50		—55	—60
3,4-Dihydroxycinnamic acid	—3		—52		—47		—67	—80
3-Methoxy-4-hydroxy-cinnamic acid			—38		—47		—64	—64
2-Hydroxy-4-methoxy-cinnamic acid	—6		—33		—39		—56	—56
Aesculin	—7		—6	—8	—36	—35	—71	—89
6,7-Dihydroxycoumarin			—7		—24		—56	
4-Hydroxycinnamic acid					—21		—63	—64
6-Methoxy-7-hydroxy-coumarin					—12		—50	—49
Xanthine	—5			—12	—11		—65	
7,8-Dihydroxycoumarin					—10		—49	—51
Umbelliferone (7-hydroxy-coumarin	—3		—3		—6		—51	—58
Inosine					—2		—27	—61
Djenkolic acid	0				0		—62	
2-Thiolhistidine	0		0	0	0		—44	
Homocystine	0				0		—22	
Ergothioneine	0		0	0	0		0	
7-Methoxycoumarin	0		0		0		0	0

to 88 in Table IV. Nevertheless, the data of WADE AND MORGAN can be used to supplement Table IV, in the pH 3.3 buffer, simply by multiplying their M_0 values by 100 to give *Am* values. Since they measured mobility from the starting line, their values do not include a correction for electro-osmotic flow, and should be lower than *Am* values. Discrepancies such as that for fructose-diphosphate may reflect the greater care taken by WADE AND MORGAN in using formic acid-washed paper to

TABLE IV

PHOSPHATE ESTERS

(In order of decreasing negative *Am* values in 9.3 borate buffer)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
Flavin-adenine dinucleotide	—68	—85	—85	—84	—88		—91	—121
Riboflavin-5-phosphate(Na)	—66	—66	—72	—69	—84	—91	—102	—119
Fructose-1,6-diphosphate(Ba)	—88	—86	—82	—82	—87	—109	—109	—92
O-Phosphoserine	—60		—66		—74		—95	—90
Uridylic acid	—58		—63	—63	—77		—93	—70
Guanylic acid	—46		—56	—59	—74		—89	—70
5′-Adenylic acid	—25		—49	—50	—65		—77	—67
Deoxycytidylic acid	—10		—47	—54	—70		—82	—63
3′-Adenylic acid	—31		—51	—53	—69		—81	—63
Glucose-1-phosphate (K)			—55	—65	—72	—84	—84	—62
Deoxyadenylic acid	—22		—49	—53	—64		—80	—61
Cytidylic acid	—14		—52	—54	—70		—84	—60
Glucose-6-phosphate (Ba)	—59	—54		—53	—61	—70	—78	—60
O-Phosphoethanolamine	—1		—10		—33		—57	—58

remove polyvalent ions that might form complexes with phosphates, and also the fact that they used only sodium salts, not barium or calcium salts. Another difference is the lower Lewis ionic strength (0.025) of the buffer used by WADE AND MORGAN, compared to the value of 0.09 for the standard pH 3.3 buffer used in our work.

The mobility pattern of a simple phosphate ester indicates a single negative charge in the range from pH 3.3 to 5.9, the *Am* value per charge being of the order of 50 for compounds of relatively low molecular weight. Mobility rises at pH 7.2 (which is near the apparent pK_2 in 30% formamide), and is maximal at pH 9.3, with 2 negative charges and an *Am* value per charge of the order of 40. If mobility increases in the pH 9.3 borate buffer, this suggests a polyol phosphate; if it decreases a cyclic sugar phosphate. If there is a striking fall in mobility in the pH 3.3 buffer, this indicates that a weakly basic group (such as adenine) is acquiring a positive charge; if mobility is very low at pH 5.9 and lower, a strong basic group is indicated. The presence of two phosphate groups raises the *Am* value but does not alter the pH–mobility pattern; this will probably also be true for the pyrophosphate esters.

Mobility of amphoteric compounds

Table V lists compounds that have a negative charge at pH 9.3 but a positive charge at pH 3.3, in order of decreasing *Am* values at pH 9.3. These are mostly neutral

TABLE V

COMPOUNDS HAVING BOTH NEGATIVE AND POSITIVE CHARGES IN NON-BORATE BUFFERS

(In order of decreasing *Am* values in pH 9.3 acetate buffer)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
Glycyl-aspartic acid	8		—42		—59	—67	—101	
Glycyl-glutamic acid	17		—32		—58	—67	—96	
Glycyl-glycyl-glycine	30						—64	—66
meso-Lanthionine	5						—63	
Acetyl-histidine	27		0	0	—32	—57	—59	
Alanyl-glycyl-glycine	32				—3	—23	—59	
Glycyl-glycyl-glycyl-glycine	30				0	—21	—58	
Alanyl-asparagine	22			5	—2	—17	—57	
Glycyl-methionine	27				0	—17	—54	
Glycyl-tyrosine	26				0	—16	—52	
Glycyl-proline	28				0	—9	—49	
Glycyl-histidine	72		63	52	22	2	—49	
Leucyl-tyrosine	23				—3	—22	—48	
Histidyl-histidine	97		69	53	—11	—18	—45	
DL-*allo*-Cystathionine	6						—44	
Carnosine	64	61	59	50	23		—29	
Serine	3						—28	—51
Isoguanine	54	21	—2			—8	—23	
3,4-Dihydroxyphenylalanine	6						—22	—56
Glycine	7		0		0		—12	—37
Alanine	5						—10	—22
Histidine	68		63		8		—10	—33
Guanine	8					—2	—10	
Isocytosine	59		1	—3			—10	
β-Alanine	61	22	14				—3	—15
Adenine		22	8			0	—2	

amino acids and peptides, since the more strongly basic or acidic ones fall in Tables I and III. Resolution and characterization of neutral peptides, like that of neutral amino acids, is poor except when additional ionizing groups give informative pH–mobility patterns.

Mobility of dinitrophenyl derivatives

Table VI lists *Am* values in order of decreasing negative *Am* at pH 9.3 of impure

TABLE VI

DINITROPHENYL DERIVATIVES OF AMINO ACIDS AND PEPTIDES

(In order of decreasing negative *Am* values at pH 9.3; values from crude preparations high in salt are always slightly below pure solution values)

Substance	*pH* 3.8	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3*B*
DNP-aspartic acid	—25		—80		—97		—96	—98
DNP-2,4-diaminobutyric acid	0		0		—81		—80	—83
DNP-glycyl-aspartic acid	—15		—70		—82		—82	—82
DNP-glycyl-glutamic acid	—12		—68		—80		—81	—79
DNP-amino-methylene-sulfonic acid	—34		—72		—70		—69	—70
DNP-djenkolic acid	—10		—39		—38		—70	—69
DNP-glycine	—48		—65		—68		—66	
DNP-taurine	—34		—65		—63		—65	
DNP-α-alanine (pure)	—48		—67		—65		—62	
DNP-α-alanine (crude)	—48		—61		—61		—58	—63
DNP-β-alanine	—11		—51		—58		—63	—62
DNP-allyl-glycine	—40		—55		—59		—61	—58
DNP-serine	—47		—62		—61		—60	
DNP-α-aminobutyric acid	—48		—56		—59		—60	—60
DNP-γ-aminobutyric acid	—4		—39		—56		—60	—58
DNP-α-aminoisobutyric acid	—47		—55		—56		—58	—59
DNP-proline	—47		—55		—57		—58	—58
DNP-threonine	—45		—53		—56		—55	—56
DNP-alanyl-leucine	—41		—55		—55		—55	—56
DNP-hydroxyproline	—48		—53		—53		—56	—55
DNP-methionine sulfoxide	—52		—50		—52		—57	—55
DNP-methionine	—45		—53		—54		—54	—55
DNP-isoleucine	—42		—52		—54		—56	—54
DNP-methionine sulfoximine	—30		—52		—52		—54	—53
DNP-glycyl-proline	—24		—46		—50		—50	—51
DNP-phenylalanine	—43		—48		—48		—48	—51
DNP-α,ε-diaminopimelic acid	—14		—41		—41		—49	—55
DNP-ε-aminocaproic acid	—2		—30		—50		—49	—50
DNP-histidine	0		0		—32		—48	—48
DNP-glycyl-tyrosine	—21		—40		—45		—46	—45
DNP-glycyl-phenylalanine	—22		—44		—44		—45	—45
DNP-alanyl-glycyl-glycine	—18		—44		—43		—43	—44
DNP-tryptophan	—31		—41		—41		—44	—44
DNP-glycyl-histidine	—5		—32		—33		—43	—44
DNP-glycyl-tryptophan	—17		—37		—42		—41	—42
DNP-δ-hydroxylysine	—15		—37		—36		—37	—39
DNP-leucyl-tyrosine	—16		—38		—37		—36	—38
DNP-lysyl-glycine	—6		—32		—32		—38	—36
DNP-galactosamine	0		0		0		0	—31
DNP-glucosamine	0		0		0		0	—28

dinitrophenyl derivatives of various amino acids and peptides. These tend to be somewhat lower than the values for pure derivatives. The pH-mobility patterns are relatively uninformative, and formation of dinitrophenyl derivatives is of limited value in characterization of unknowns by ionophoresis.

Mobility of some inorganic ions

Table VIII lists a few ions, in order of decreasing *Im* value at pH 3.3. Values are of the expected order of magnitude in solutions of high ionic strength. It is interesting to note that calcium and magnesium ions move well even in the pH 9.3 buffer, presumably because the 30% formamide maintains the ionization of their hydroxides.

TABLE VIII

INORGANIC IONS

(In order of decreasing *Im* values at pH 3.3)

Substance	*pH* 3.3	*pH* 4.0	*pH* 4.7	*pH* 5.9	*pH* 7.2	*pH* 8.0	*pH* 9.3	*pH* 9.3B
Potassium	192	190	195	218	217	210	223	160
Sodium	151	150	157	157	159	158	160	135
Calcium	135	130					113	
Magnesium	121	110					106	
Manganese	104	100						
Cobalt	103	98						
Nickel	90	89						
Phosphate	−100	−99	−97	−99	−98	−100	−135	−89

Sodium, potassium, phosphate, and probably other soluble ions show a striking relative mobility depression in the pH 9.3 borate buffer, presumably caused by the very high Lewis ionic strength (0.38) of this buffer.

Influence of buffer viscosity on ionic mobility

The important factors determining the mobility of ions are the ionic strength and the viscosity of the buffer. The viscosity of 30% formamide (relative to water) is 1.13. Most of the organic buffers increase the viscosity to about 1.2, but the more concentrated pH 9.3 borate buffer has a relative viscosity of 1.34. In this buffer the absolute mobility of Amaranth is only about 0.35 mm/h, and the mobility of Brilliant Blue is 50 *Im* units. If the borate buffer is diluted with three volumes of 30% formamide, its viscosity and ionic strength become comparable to the other standard buffers. The absolute mobility of Amaranth, however, rises to 0.55 mm/h (compared to about 0.45–0.5 mm/h in the other buffers), and the relative mobility of Brilliant Blue rises to 59 *Im* units (compared to 54–63 units in the other buffers).

A similar depression both of absolute mobilities and of the *Im* value of Brilliant Blue has been noted when a more concentrated pH 8 (N-ethylmorpholine acetate) buffer was compared with the standard buffer, and it is clear that the ionic strength can significantly influence *Im* values.

SUMMARY

The *Am* values of many known compounds in 30% formamide organic buffers at several pH values have been tabulated to aid in comparison and identification of unknowns. The p*K* and molecular weight values calculable from ionophoretic data sometimes differ considerably from expected values because of unusually strong molecular interactions with the formamide buffers. The mobility–pH pattern nevertheless gives significant information about molecular structure of unknowns.

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A PROCEDURE FOR THE ANALYSIS OF ACID-SOLUBLE PHOSPHORUS COMPOUNDS AND RELATED SUBSTANCES IN MUSCLE AND OTHER TISSUES

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(Received November 14th, 1960)

In 1956, THRELFALL[1] published a procedure enabling him to separate and estimate quantitatively 10 different phosphorus compounds present in an acid extract of rat muscle. The chromatographic methods used to separate these substances on paper were described only briefly. The object of the present work has been to enlarge and extend the methods of THRELFALL[1] and other workers so as to develop a simple but comprehensive procedure for investigating phosphorus compounds in aqueous tissue extracts. Procedures for the water-soluble non-phosphorylated compounds have already been described by the author[2-4].

MATERIALS AND EQUIPMENT

Chromatography was carried out with the "Universal Chromatography Outfit" supplied by Aimer Products Ltd.*. The apparatus was adapted by the author to eliminate all metallic parts. Trays were constructed of 1/4 in. polythene sheet welded together at the edges; the frames consisted of 1/4 in. polythene sheet joined by four 8 mm diameter glass rods, and the spacers were of 1 1/4 in. lengths of polythene tubing, 3/8 in. internal diameter. All chromatograms were of the ascending type on 10 in. square sheets of Whatman No. 541 paper. Norit SX 30 Special was a gift from Swift & Co. Ltd., Sydney; it was washed three times with 10% (v/v) aqueous pyridine to remove ultraviolet-absorbing impurities, then several times with water and dried at 110°. Celite 535 was obtained from Johns Manville Co. Ltd., London. The "Chromatolite" was purchased from Hanovia Ltd., Slough, Bucks.

Reference compounds

Acetyl phosphate (Ac-P), creatine phosphate (Cr-P), carbamyl phosphate (Carb-P), and phospho-enol-pyruvate (PEP), were the synthetic compounds obtained from Dr. G. M. KELLERMAN of this department. Ethanolamine-O-phosphate (Et-P), choline phosphate (Ch-P), and serine phosphate (Se-P), were synthetic compounds, the gift of Mr. H. G. WESTALL, University College Hospital Medical School, London.

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Glycerylphosphorylcholine (GPC) was the gift of Dr. I. G. WHITE who obtained it from Dr. R. M. C. DAWSON, Institute of Animal Physiology, Babraham Hall, Cambridge. Glycerol-1-phosphate (Gl-1-P) was prepared from the 2-phosphate by the method of BAILLY[5]. Uridine diphosphate glucose (UDPG) was a gift from Dr. J. F. TURNER, C.S.I.R.O. Plant Physiology Unit, University of Sydney. The flavinadenine dinucleotide (FAD) was from yeast and was supplied by Dr. A. W. LINNANE of this department. Sedoheptulose-7-phosphate (S-7-P) and ribulose-1,5-diphosphate (Ru-1,5-PP) were the gift of Professor B. L. HORECKER, Department of Microbiology, New York University College of Medecine. Phytic acid (PA) was donated by Dr. J. W. LEE, C.S.I.R.O. Wheat Research Unit, Bread Research Institute of Australia, N. Ryde, N.S.W. Adenosine diphosphate ribose (ADP-R) and adenosine triphosphate ribose (ATP-R) were the main products of the action of 3 % perchloric acid on DPN and TPN respectively for 30 min at room temperature[6]. 2,3-Diphosphoglyceric acid (2,3-PPGA) was the principal phosphate present in the barium-insoluble fraction of rat blood[7,8]. Adenylsuccinic acid (AMPS) was prepared by the method of DAVEY[36] and propane-1,2-diol-1-phosphate (PDP) was synthesized as described by RUDNEY[37]. Diphosphopyridine nucleotide (DPN) and the reduced nucleotide (DPNH), triphosphopyridine nucleotide (TPN) and the reduced nucleotide (TPNH), adenosine-3′-, and -5′-phosphates (AMP-3′ and AMP-5′), adenosine diphosphate (ADP), adenosine triphosphate (ATP), inosine monophosphate (IMP), diphosphate (IDP) and triphosphate (ITP), uridine-3′- and -5′-phosphates (UMP-3′ and UMP-5′), uridine diphosphate (UDP) and triphosphate (UTP), cytidine-3′- and -5′-phosphates (CMP-3′ and CMP-5′), cytidine diphosphate (CDP) and triphosphate (CTP), guanosine-3′- and -5′-phosphates (GMP-3′ and GMP-5′), guanosine diphosphate (GDP) and triphosphate (GTP), flavin mononucleotide (FMN), thiamine pyrophosphate (TPP), fructose-1-phosphate (F-1-P), fructose-6-phosphate (F-6-P), glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-1,6-diphosphate (F-1,6-PP), ribose-5-phosphate (R-5-P), 3-phosphoglycerate (3-PGA), 2-phosphoglycerate (2-PGA), glycerol-2-phosphate (Gl-2-P), galactose-6-phosphate (Ga-6-P), mannose-6-phosphate (M-6-P), 6-phosphogluconate (6-PG), dihydroxyacetone phosphate (DHAP), and glyceraldehyde-3-phosphate (GAP), pyridoxal phosphate (Py-P), adenine (Ad), adenosine (Ao), hypoxanthine (Hy), inosine (I), guanine (Gu), glucose (G), fructose (F), ribose (R), galactose (Ga), and maltose (Ma), were the purest obtainable commercially. Solutions were made in water or 10 % (v/v) isopropanol and stored at 2° or —10° depending on the lability of the compound.

EXPERIMENTAL METHODS AND RESULTS

Preparation of extracts

10 to 20 g of tissue were extracted with 3 % (v/v) perchloric acid, rapidly filtered and the filtrate neutralised with 10 % (w/v) potassium hydroxide. These operations were carried out in the cold-room at 2°. The neutralised filtrate was stood at least 4 h, or overnight, at 2° to allow precipitation of potassium perchlorate to occur.

The solution was filtered and freeze-dried. When the volume of extract was greater than 20 ml, further amounts of potassium perchlorate were removed by redissolving the freeze-dried material in 10 to 15 ml of water, filtering and freeze-drying the filtrate. The freeze-dried extracts were stored over phosphorus pentoxide *in vacuo* at 2° and solutions were kept frozen at —10°.

Removal of calcium and magnesium ions

It was thought desirable to remove calcium and magnesium ions which are known to form complexes with phosphate esters and interfere with their chromatography[9]. THRELFALL[1] used Amberlite IR-120 (H^+) for this purpose. The equivalent resin, Zeo-Karb 225 (H^+) was tested but chromatography of the extracts before, and after, treatment with the resin showed that some of the phosphates had been removed in addition to the cations. A column of Zeo-Karb 226 (NH_4^+) gave better results and development of chromatograms of the effluent with 8-hydroxyquinoline[10] showed the removal of calcium ions without loss of phosphates. When barium precipitation was to be used subsequently to separate the phosphates, the removal of calcium and magnesium was omitted.

Barium fractionation

The procedure described by GERLACH AND WEBER[11] was followed, but omitting the reprecipitation of each fraction. The compounds appearing in fractions A (barium-insoluble), B (barium-soluble, alcohol-insoluble), and C (barium-soluble, alcohol-soluble) agreed fairly well with the distribution reported by these authors with the exception of DHAP which sometimes appeared in fraction A instead of B, F-1, 6-PP which tended to appear in fraction B instead of A, and DPN which sometimes precipitated in fraction B but most often remained in fraction C. The occurrence of any one compound in a particular fraction appeared to depend on the exact conditions and on the other compounds present, so this information was only used as an *indication* of the nature of that compound.

To prepare solutions for chromatography, the barium precipitate was resuspended in water and stirred with either Zeo-Karb 225 (H^+) or Zeo-Karb 226 (NH_4^+). Treatment with Zeo-Karb 226 did not result in the liberation of acid but there was some danger that the barium salts would not be completely decomposed under these conditions and sometimes, on centrifuging, a small amount of undecomposed material could be seen lying on top of the resin. When Zeo-Karb 225 was used, there was little likelihood of the precipitate remaining undissolved but a distinct possibility that any acid-labile phosphates would be decomposed by the acid liberated before this could be neutralised after removal of the resin. Also, as mentioned previously, it was found that some organic phosphates could adsorb onto Zeo-Karb 225, so, in general, treatment with Zeo-Karb 226 was preferred. If, after 5 to 10 min stirring, the suspended barium precipitate had not all been decomposed, more resin was added. After centrifuging, the supernatant was removed with a pipette, the pH adjusted and the solution freeze-dried. The alcohol was removed from fraction C at 40° under reduced pressure, the barium removed with ion-exchange resin and the solution freeze-dried.

Paper chromatography

The freeze-dried material, dissolved in a minimum amount of water, was applied to the paper by a platinum wire loop, and the spot dried by a blast of cold air from a hair drier. 0.6 g of ethylenediaminetetraacetic acid was added to each 200 ml of solvent placed in the tray.

Solvents

(1) Isobutyric acid–*N* ammonium hydroxide (100:60 by vol.)[12].

(2) *n*-Propanol–0.880 ammonium hydroxide–water (60:30:10 by vol.)[9].

(3) *n*-Butanol–acetic acid–water (60:15:25 by vol.)[13].

The following two solvents were chosen from the five described by GERLACH, WEBER AND DÖRING[14], and are designated GW1 and GW3 respectively. Both were used twice in the same direction.

(GW1) Isopropyl ether–*n*-butanol–90% (w/w) formic acid* (30:30:20 by vol.).

(GW3) *n*-Butanol–*n*-propanol–acetone–80% (w/w) formic acid–30% (w/v) trichloroacetic acid (40:20:25:25:15 by vol.).

The above solvents were selected from the many described in the literature and each had its own particular virtues. Cr-P was stable in propanol–ammonia, so this solvent was used for its separation from creatine and detection with diacetyl. The solvent GW1 left nucleotides and most sugar phosphates at the origin and was suitable for the detection of three-carbon phosphates. GW3 had the great advantage of producing a good spread of compounds across the paper and of being able to move large amounts of orthophosphate; only two phosphates ran further than orthophosphate in this solvent. The butanol–acetic acid solvent was chosen since the R_F values of many muscle constituents in this solvent have already been determined[13].

Many investigators have observed the appearance of two or more spots when orthophosphate was run on paper chromatograms. CURRY[15] investigated the behaviour of orthophosphates and free phosphoric acid on chromatography and concluded that the several spots observed were due to different ionic forms running independently. With the solvents used here, with the exception of butanol–acetic acid, multiple spots of phosphates were not observed. The addition of 1% (w/v) boric acid to the propanol–ammonia and isobutyric acid solvents lowered the R_F values of sugar phosphates containing *cis*-vicinal hydroxyl groups, as described by COHEN AND SCOTT[16] and by HARRAP[17], thus separating them from other compounds of similar R_F not possessing this grouping. An example is the pair G-1-P and G-6-P; addition

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[*] The solvent described by the authors contained 98–100% formic acid but the solvent described here was found to be very satisfactory.

Solvents 1 and 2 were stable for some weeks; others were rejected after 4 to 5 days. In some experiments, 0.6 g of A.R. boric acid was added to 60 ml of the propanol–ammonia or the isobutyric acid solvents.

of boric acid decreased the R_F of G-6-P in propanol–ammonia from 0.26 to 0.16 whereas that of G-1-P was not affected.

The R_F, R_{PO_4} and R_{AMP} values of a number of phosphates in the above solvents were determined by running about 30 compounds on five sheets of paper, simultaneously, in each solvent. Although the absolute values varied from run to run, the relative positions of the compounds remained constant and were checked over a period of two years while this work was in progress, during which time over 600 chromatograms were run. The values found are listed in Table I.

TABLE I

R VALUES OF PHOSPHATES AND RELATED COMPOUNDS

All values determined on Whatman No. 541 paper; values in GW3 twice ($GW3^2$) and GW1 twice ($GW1^2$) are R_{PO_4} values, and in isobutyric acid are $R_{AMP\text{-}5'}$ values. Decomposition in a particular solvent is indicated by "decomp".

Compound	Solvents				
	$GW3^2$ R_{PO_4}	1 $R_{AMP\text{-}5'}$	$GW1^2$ R_{PO_4}	2 R_F	3 R_F
AMP-5′	0.70	1.00	0.00	0.26	0.20
AMP-3′	0.80	1.22	0.00	0.30	0.22
ADP	0.24	0.82	0.00	0.18	0.05
ATP	0.12	0.75	0.00	0.15	0.04
IMP	0.49	0.58	0.00	0.17	0.17
IDP	0.19	0.43	0.00	0.13	0.09
ITP	0.07	0.35	0.00	0.10	0.05
UMP-3′	0.63	0.69	0.03	0.20	0.24
UMP-5′	0.58	0.60	0.01	0.15	0.20
UDP	0.24	0.45	0.00	0.14	0.07
UTP	0.09	0.36	0.00	0.13	0.06
GMP-3′	0.56	0.70	0.00	0.12	0.18
GMP-5′	0.51	0.56	0.00	0.10	0.18
GDP	0.05	0.39	0.00	0.07	0.05
GTP	0.02	0.26	0.00	0.06	0.02
CMP-3′	0.64	0.95	0.01	0.26	0.20
CMP-5′	0.58	0.85	0.00	0.20	0.17
CDP	0.22	0.65	0.00	0.14	0.08
CTP	0.07	0.52	0.00	0.11	0.03
UDPG	0.13	0.25	0.00	0.60	0.05
DPN	0.10	1.02	0.00	decomp.	0.04
DPNH	decomp.	decomp.	0.00	decomp.	decomp.
ADP-R	0.10	0.73	0.00	0.30	0.04
AMPS	0.70	0.62	0.00	0.17	0.25
TPN	0.05	0.71	0.00	decomp.	0.01
TPNH	decomp.	decomp.	0.00	decomp.	decomp.
ATP-R	0.07	0.48	0.00	0.12	0.01
FMN	0.59*	0.67	0.00	0.55*	0.08*
FAD	0.67*	0.75	0.00	0.53*	0.00*
Py-P	1.05	0.80	0.18	0.23	0.26
TPP	0.52	1.26	0.00	decomp.	0.10
PO_4	1.00	0.68	1.00	0.19	0.28
SO_4	0.46	0.44	0.02	0.17	0.08
P_2O_7	0.52	0.52	0.02	0.11	0.11
Cr-P	0.98*	0.86*	0.62*	0.17	decomp.
Ac-P	decomp.	0.86*	decomp.	decomp.	decomp.

(continued on p. 147)

TABLE I (*continued*)

Compound	*Solvents*				
	GW3^2 R_{PO_4}	1 $R_{AMP\text{-}5'}$	GW1^2 R_{PO_4}	2 R_F	3 R_F
Carb-P	0.46*	0.40	decomp.	decomp.	decomp.
GAP	0.72	0.40	0.23	decomp.	0.15
DHAP	0.70	0.68	0.23	decomp.	0.05
PDP	1.19	0.95	1.33	0.33	0.28
PEP	1.06	0.62	0.73	0.26	0.27
3-PGA	0.79	0.45	0.32	0.23	0.18
2-PGA	0.74	0.45	0.20	0.26	0.16
2,3-PPGA	0.54	0.38	0.01	0.04	0.12
Gl-1-P	0.76	0.65	0.52	0.35	0.14
Gl-2-P	0.83	0.65	0.52	0.35	0.14
Et-P	0.81	0.89	0.08	0.30	0.21
Se-P	0.67	0.54	0.02	0.04	0.17
Ch-P	0.90	1.06	0.23	0.33	0.24
GPC	0.67	1.01	0.12	0.49	0.28
R-5-P	0.57	0.53	0.04	0.25	0.18
F-1-P	0.52	0.40	0.01	0.27	0.12
F-6-P	0.50	0.48	0.03	0.31	0.19
F-1,6-PP	0.35	0.37	0.01	0.09	0.08
G-1-P	0.41	0.48	0.02	0.30	0.20
G-6-P	0.41	0.46	0.02	0.26	0.12
6-PG	0.40	0.42	0.02	0.22	0.12
Ga-6-P	0.41	0.46	0.02	0.25	0.12
M-6-P	0.47	0.51	0.02	0.31	0.14
S-7-P	0.45	0.46	0.00	0.28	0.12
Ru-1,5-PP	0.30	0.35	0.00	0.06	0.08
PA	0.07	0.15	0.00	0.01	0.01
G	0.59	0.66	0.16	0.77	0.22
F	0.68	0.77	0.21	0.98	0.26
R	0.86	0.84	0.40	0.78	0.30
Ga	0.55	0.66	0.16	0.73	0.22
Ma	0.32	0.55	0.00	0.65	0.14
Ad	1.40	1.60	0.30	0.72	0.60
Hy	1.15	1.10	0.31	0.66	0.48
Gu	0.78	1.20	0.14	0.45	0.38
Xo	1.00	1.57	0.16	0.84	0.49
I	0.75	0.96	0.16	0.65	0.36

* Partial decomposition but *R* value of intact portion quoted.

The values in GW3 twice and GW1 twice showed good agreement with those published by GERLACH, WEBER AND DÖRING[14]. The nucleoside triphosphates were stable in all solvents, Cr-P was partially decomposed by all solvents except propanol–ammonia, while DPN, DPNH, TPN, and TPNH were decomposed by this solvent to give a substance fluorescing blue under ultraviolet light. DPNH and TPNH were decomposed by acid solvents forming ADP-R and ATP-R respectively as described by FORREST, WILKEN AND HANSEN[6]; the decomposition of DPNH and TPNH by acid has also been discused by RAFTER, CHAYKIN AND KREBS[18]. FAD and FMN were slowly decomposed by both acid and alkaline solvents as would be expected from their known behaviour in acid and alkali[19] but they seemed to be stable in isobutyric acid. GAP and DHAP were stable in the acid solvents but decomposed by propanol–ammonia.

Two-dimensional chromatography

Different combinations of solvents were found suitable for different purposes. For the separation and identification of the nucleotides, isobutyric acid followed by GW3 twice was the best combination. It was discovered that when ATP was run with GW3 as the first solvent, the chromatograms showed an additional spot due to small amounts of ADP. When, however, the order of the solvents was reversed only the single spot of ATP resulted. Since only a single spot resulted when ATP was run in GW3 alone, it was concluded that the deposition of trichloracetic acid on the paper on drying, after the second run in GW3 and prior to the run in isobutyric acid, had lowered the pH of the paper to a point where some of the ATP had hydrolysed. Therefore, as a general rule, when the identification of nucleotides was the aim, GW3 was used as the *second* solvent. However, when reducing sugar phosphates were to be detected with the triphenyltetrazolium reagent, better results were obtained when the solvents were used in the reverse order since the isobutyric acid washed to one side the trichloracetic acid deposited on the paper which otherwise interfered considerably with the colour development of the spots. The combination GW3 twice/GW1 twice was found very useful for separating three-carbon phosphates, since the nucleotides, pentose phosphates, and hexose phosphates remain very near the origin in the GW1 direction. "Maps of the spots" of the phosphates most likely to be found in animal tissues are shown in Figs. 1 and 2.

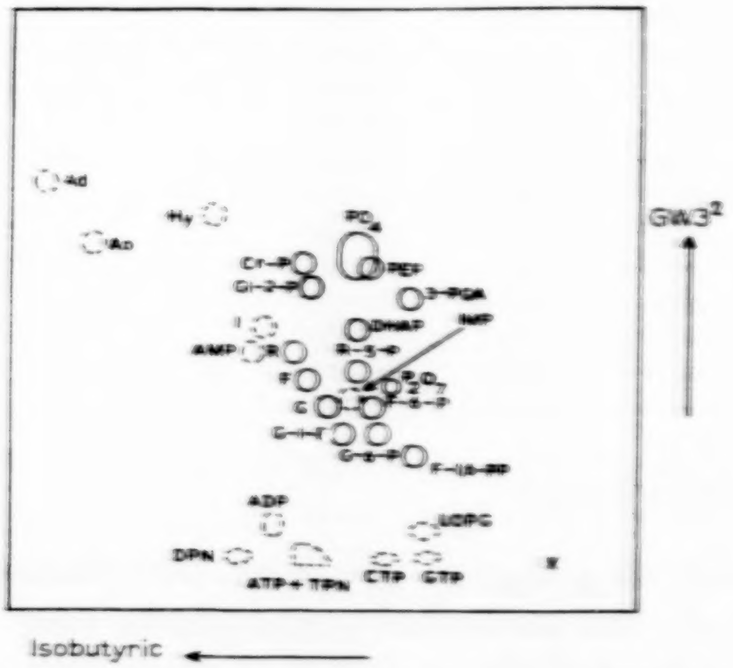

Fig. 1. "Map of the spots" on chromatograms in GW3 twice followed by isobutyric acid. Origin marked by a cross; spots visible under ultraviolet light are shown in broken outline.

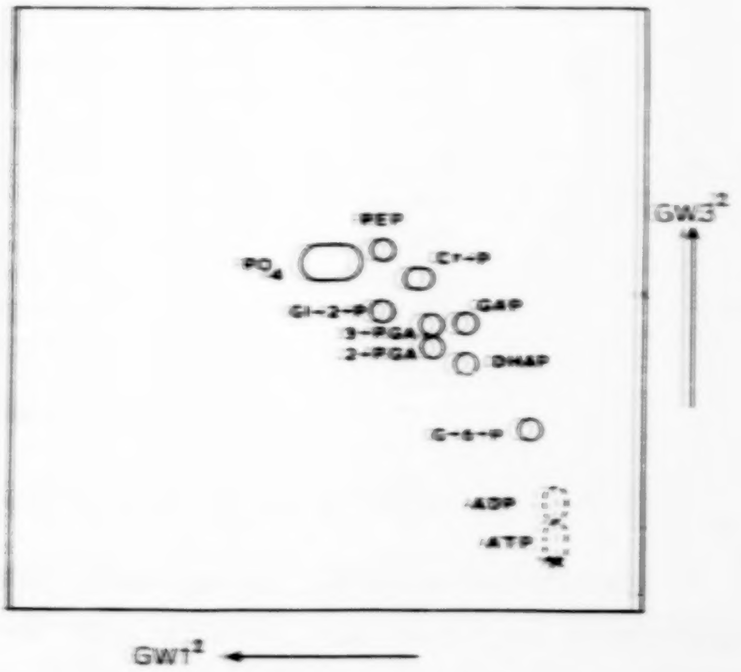

Fig. 2. "Map of the spots" on chromatograms in GW3 twice followed by GW1 twice. Origin marked by a cross; spots visible under ultraviolet light are shown in broken outline.

DETECTION OF COMPOUNDS ON CHROMATOGRAMS

Ultraviolet light

Chromatograms were viewed under ultraviolet light from a Hanovia "Chromatolite". Dark spots of purine and pyrimidine derivatives and any fluorescent spots were marked. Guanine derivatives showed a dark blue fluorescence coincident with the dark spot when acid solvents had been used. To make this more apparent, or when the last solvent was not strongly acidic, the paper was first dipped through a solution of 0.05 ml concentrated hydrochloric acid in 50 ml acetone. After chromatography in GW$_3$ twice isobutyric acid and leaving the papers overnight in the warm room at 30°, GAP, DHAP, and R-5-P gave faint yellow or blue fluorescent spots under the lamp. Since these spots were not visible directly after the solvent had been removed it was concluded that the fluorescence was due to reaction with the ammonium isobutyrate deposited on the paper to form a fluorescent "Maillard-type" compound[20].

Acid molybdate

The reagent of Hanes and Isherwood[9] as modified by Burrows, Grylls and Harrison[21] was used and the spots were developed by exposure to ultraviolet light. After dipping, and before irradiation, orthophosphate and very labile compounds such as Cr-P, Ac-P, Carb-P, GAP, and DHAP, always showed as yellow spots. ATP and other nucleoside triphosphates sometimes appeared as bright yellow spots and ADP and other nucleoside diphosphates were also faintly yellow. This only occurred when GW$_3$ was used as the final solvent; apparently the increased acidity due to the trichloracetic acid deposited on the paper after drying was sufficient to bring about liberation of orthophosphate at room temperature on treatment with acid-molybdate. The effect could be increased by diluting the reagent with a smaller volume of acetone before applying it to the paper. G-1-P sometimes behaved in a similar manner; it could be distinguished from G-6-P, which runs very close to it on two-dimensional chromatograms, by the fact that the blue spot brought up on irradiation always had a yellow-green colour when viewed from the reverse unirradiated side of the paper.

Despite the fact that it was slowly decomposed by the solvents used, quite large spots of Cr-P were found on two-dimensional chromatograms of muscle extracts. At first, it was thought that orthophosphate had split into two spots but a strong reaction with diacetyl showed that the spot was, in fact, intact Cr-P. Pyrophosphate did not give a spot until after irradiation and the spot was blue. The yellow colour of the background produced on irradiation slowly returned to white as moisture was regained from the air. Alternatively, the paper was exposed momentarily to steam; this treatment also increased the brightness of the blue spots. Sulphate ion could give a spurious blue spot if present in great enough quantity so its R values are quoted in Table I.

Adenine compounds

The procedure of Gerlach and Döring[22] showed Ad, Ao, AMP, ADP, ATP, and the pyridine nucleotides, as red spots; other nucleotides gave no colour.

Pyridine nucleotides

These were detected with methyl ethyl ketone and ammonia as described by Kodicek and Reddi[23].

Thiamine pyrophosphate

TPP was detected by dipping the paper through the reagent described by Block, Durrum and Zweig[24]. After drying, TPP was revealed as a bright blue fluorescent spot under ultraviolet light. The reaction was not given by the pyridine nucleotides.

Silver nitrate–bromophenol blue

As an additional reagent for purine and pyrimidine compounds this was particularly useful for confirming the presence of inosine and hypoxanthine[25].

Cations

The procedure of Sommer[10] revealed Mg^{++} and Ca^{++} as fluorescent spots under ultraviolet light.

Diacetyl

The diacetyl reagent of Foster and Ashton[26] was used to detect creatine and Cr-P. The reagent did not react directly with Cr-P which had to be broken down by heating the papers in an oven for a few minutes at 105° before treatment with the reagent.

Diphenylamine

The reagent described by Stepanenko and Kuznetsov[27] gave a very characteristic rose colour with F-1,6-PP but was of rather low sensitivity.

p-Anisidine

The reagent of Pridham[28] was adapted for dipping. 2.5 g *p*-anisidine hydrochloride and 0.25 g sodium bisulphite were added to 50 ml methanol followed by a few drops of water sufficient to dissolve the bisulphite. Before use the solution was diluted 5 times with acetone. After dipping, the papers were dried and heated at 130° for a few minutes. Glucose, fructose, and their various phosphates gave brown spots while ribose and R-5-P gave red-purple spots.

Orcinol

The reagent described by Klevstrand and Nordal[29] was used to detect heptose phosphates. By its use S-7-P was detected in liver extracts.

2,4-Dinitrophenylhydrazine

The paper was drawn rapidly through the surface of a saturated solution of the reagent in 2 *N* hydrochloric acid, taking care not to waterlog the paper. If present in sufficient amount, GAP and DHAP appeared immediately as orange spots on a yellow background.

Pyrophosphate reagent

1.2 g cobalt chloride hexahydrate were dissolved in 12 ml of water, acidified with a few drops of 2 *N* acetic acid, and 28 ml of ethanol added. The papers were dipped through the reagent, dried, and the background colour removed by two washes in 50% (v/v) ethanol. After drying, the still humid papers were exposed to ammonia fumes and pyrophosphate appeared as a brown spot. Sensitivity: 5 μg P as pyrophosphate.

Triphenyltetrazolium

Before applying the reagent[30] the chromatograms were hung in the air for 6 to 7 days to remove traces of solvent which otherwise caused considerable background colour. The papers were dipped through a freshly prepared solution of 0.1 g 2,3,5-triphenyltetrazolium chloride in 20 ml chloroform, dried, and then passed through a solution of 20% (w/v) aqueous potassium hydroxide diluted to 2% (w/v) with *n*-butanol–ethanol (1:1). The papers were dried in the dark and spots of very strongly reducing sugar phosphates such as R-5-P showed up at room temperature, if present in sufficient amount. The papers were exposed to steam from a boiling water-bath to bring up the reducing sugars or sugar phosphates as red spots on a white or pink background. The background colour could be diminished by careful washing in 50% (v/v) ethanol.

The following substances reacted: glucose, fructose, ribose, galactose, maltose, GAP, DHAP, R-5-P, F-1-P, F-6-P, G-6-P, Ga-6-P, M-6-P, S-7-P, Ru-1,5-PP, DPN, DPNH, TPN, TPNH, ADP-R, ATP-R, and UDPG. G-1-P did not react, and F-1,6-PP gave only a weak reaction.

QUANTITATIVE MEASUREMENT

(a) By measurement of ultraviolet absorption

The spot, as revealed under ultraviolet light, was cut out and placed in a clean dry test-tube. After washing twice with A.R. methanol the tube and paper were drained and dried. 1.2 ml of 0.1 *N* hydrochloric acid were added and the tube was stoppered and left for 18 h with occasional shaking. The optical density of the eluted material was measured in a Beckman spectrophotometer over the range 240 to 290 mμ against a "blank"* prepared from a piece of paper of the same size cut from the corresponding position of a "blank" chromatogram run at the same time. The absorption curve of the eluted material was plotted and the compound identified from the wave-length of its absorption maximum and the position on the chromatogram. The amount present could be calculated from the height of the curve and the published molar extinction coefficient.

In cases where there was not sufficient material in the spot to give a measurable absorption in solution, it was found possible to cut out the spot and measure its absorption directly on the paper, as follows: the spot was moistened with glycerol and caused to adhere to the outside of the Beckman cell filled with water such that it completely covered the aperture in the cell carrier; the same was done with a "blank" prepared as above and the absorption measured over a range of wavelengths against the blank. Obviously this technique could be adapted to semi-quantitative measurement of the amount present.

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* BRINER[35] has published some useful data on the ultraviolet absorption of material eluted from paper cut from various positions of such chromatograms.

(b) By measurement of the phosphorus in a spot

After development with acid-molybdate, spots were cut out and ashed with 0.2 ml of a mixture of concentrated sulphuric acid–10 *N* perchloric acid (1:1 v/v) in a pyrex tube at a temperature not greater than 220°. The acid and washings were transferred to a 10-ml "Quickfit" stoppered test-tube bearing a mark corresponding to 3.5 ml. The solution was made up to the mark and the phosphate determined by MARTIN AND DOTY's[31] modification of the method of BERENBLUM AND CHAIN[32] as described by LINDBERG AND ERNSTER[33]. The volumes used were as follows:

0.5 ml of 10% (w/v) ammonium molybdate was added (final acid concentration approx. 1.1 *N*) and the phosphomolybdic acid was extracted with 3.00 ml benzene–isobutanol (1:1 v/v). 2.00 ml of the supernatant were removed with an all-glass "Tuberculin" syringe, 2.90 ml of 3.2% (v/v) sulphuric acid in ethanol were added followed by 0.10 ml 1% (w/v) stannous chloride and the colour was read at 725 mμ against a blank prepared from 0.2 ml of the digestion acid taken through the same procedure.

It was most important to use fresh stannous chloride which was prepared by dissolving 0.1 g granulated tin in 2 ml concentrated hydrochloric acid and diluting to 20 ml with *N* sulphuric acid. The solution was rejected when more than 5 days old.

A standard curve was prepared over the range of final concentrations 0.10–1.60 μg per ml. The optical densities were found to be quite stable and reproducible from day to day, but standards were always run together with the samples. The standard curve was linear over the above range and up to a final concentration of 8.0 μg per ml. The blank for Whatman No. 541 paper, after being used for phosphate chromatography, was found to be 0.02 μg of phosphorus per cm^2.

DISCUSSION

A large number of published methods for the isolation and separation of organic phosphates were tried and have led to the procedures described here. Many reagents for identifying the separated compounds were tested and those selected were chosen for their specificity and high sensitivity. On two-dimensional chromatograms, spots containing as little as 0.3 μg of phosphorus could be detected by the acid-molybdate reagent, and the triphenyltetrazolium reagent was, if anything, even more sensitive to such compounds as F-6-P and G-6-P.

Large amounts of material, containing a high proportion of orthophosphate, could be chromatographed in the system GW3 twice/isobutyric acid and the phosphates could be detected in extracts applied directly to the paper. However, the best results were achieved by chromatography of the 3 fractions obtained after barium precipitation, since not only were most of the phosphates thereby concentrated, but the occurrence of a compound in a particular fraction aided its identification.

The use of freeze-drying for concentration of the extracts and various fractions was essential; the ease and rapidity with which some sugar phosphates react with amino acids and ammonia has been described by the author[34]. Such reactions, if allowed to take place, would result in the non-detection of some phosphates originally present (particularly R-5-P) and the discovery of "unknown" compounds produced as intermediates in the "Maillard" reaction[20].

The methods described here are of general application and have been applied extensively to the investigation of the acid-soluble phosphates in rat muscle and ox muscle, and also to extracts of whale muscle, rat blood, and rat liver. Diagrams of the chromatograms of extracts of ox muscle will be published elsewhere[34]. It is hoped to present a full report on the compounds found in rat muscle, among which the following have been positively identified: Cr-P, ATP, ADP, GTP, DPN, F-6-P, G-1-P, F-1,6-PP, DHAP.

ACKNOWLEDGEMENT

This work was assisted financially by the Commonwealth Scientific and Industrial Research Organisation of Australia.

SUMMARY

A method for the separation, identification, and quantitative measurement of the acid-soluble phosphates and related compounds in tissues is presented. The *R* values of 70 compounds in 5 different solvents are listed and "maps of the spots" of those most commonly found in tissues are given. A new reagent for detecting pyrophosphate is described.

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DIE ZERSETZUNG DER AMINOSÄUREESTER AUF EINWIRKUNG VON HBr IN EISESSIG

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(Eingegangen den 26. November 1960)

Um eine eindeutig ablaufende Reaktion zu erhalten, werden bei der Synthese der verschiedenen Peptide jene Gruppen der bifunktionalen Aminosäuren, die in der Reaktion nicht teilnehmen sollen, bekanntlich geschützt. Zum Schutz der Carboxylgruppe bedient man sich in vielen Fällen des mit Benzylalkohol (BzOH) gebildeten Esters, während die Aminogruppe in der Regel in Form des Carbobenzoxy-Derivats (CBO-) geschützt wird[1].

Vor der Koppelung des N-terminalen Teiles eines N-CBO-dipeptid-benzylesters mit einer neuen Aminosäure muss die Schutzgruppe abgespalten werden; das zu diesem Zweck meist verwendete Verfahren ist die Behandlung mit eisessigsaurem HBr. Im Laufe unserer Versuche stellten wir fest, dass sich das bei der Reaktion von papierchromatographisch reinem Glutaminsäure-dibenzylester und *p*-Cl-CBO-Alanin gebildete N-*p*-Cl-CBO-ala-glu-$(OBz)_2$ – dessen Elementaranalyse mit den berechneten Werten übereinstimmte – nach Behandlung mit eisessigsaurem HBr papierchromatographisch nicht als einheitlich erwies, sondern mehrere Verunreinigungen in nicht zu vernachlässigender Menge enthielt. Diese Beobachtung bildete die Grundlage unserer Versuche. Die Chromatogramme führten zwangsweise zur Schlussfolgerung, dass auf Einwirkung des eisessigsauren HBr nicht nur die Schutzgruppen des N-terminalen Molekülteiles abgespalten wurden, sondern dass dabei auch die Esterbindungen weitgehend "hydrolysiert" wurden.

Zwecks Klärung dieses Problems stellten wir verschiedene Ester einiger Aminosäuren dar, behandelten diese mit eisessigsaurem HBr, chromatographierten das Produkt und führten die Auswertung der Chromatogramme nach Entwicklung mit Ninhydrin mit einem Densitometer durch. Aus den Lösungen wurde das Chromatographieren und die Auswertung nach 24-stündigem Stehen wiederholt.

Die Ergebnisse sind in Tabelle I zusammengefasst.

Aus den Daten der Tabelle I geht deutlich hervor, dass die Zersetzung einiger Aminosäureester eine weitgehende ist. Bei den präparativen Arbeiten bereiten die Nebenreaktionen ziemliche Schwierigkeiten; über diese berichteten wir an dem III. Europäischen Peptid-Symposium, 1960, in Basel[9]. Werden an Stelle der Benzylester die weniger reaktionsfähigen Äthylester verwendet, so ist das Ausmass der Zersetzung zwar geringer, jedoch noch immer bedeutend. Als das am meisten zersetzliche in der

Reihe der untersuchten Aminosäure-Derivate erwies sich das Glu-γ-OBz. Interessanterweise erscheint nach der Behandlung des Glu-α-OBz mit eisessigsaurem HBr am Papierchromatogramm auch der Fleck des Dibenzylesters – und zwar in ziemlich bedeutendem Masse. Die Erklärung dieser Erscheinung dürfte darin zu suchen sein, dass das sich im Laufe der partialen Hydrolyse des α-Esters bildende Benzylbromid fähig ist, die leicht veresterbare, sehr reaktive γ-Carboxylgruppe zu verestern. Eine ähnliche Erscheinung wurde beim α-Benzylester der Asparaginsäure nicht beobachtet.

TABELLE I

Aminosäure-Derivat	*Herstellung: Lit.*	*Prozentueller Anteil*					
		nach d. Behandlung			*nach 24 Stunden*		
		Diester	*Monoester*	*Säure*	*Diester*	*Monoester*	*Säure*
Glu(OBz)$_2$	2	42	41	17	25	42	33
Asp(OBz)$_2$	3	76	18	6	55	27	18
Glu-γ-OBz	4	0	27	73	0	10	90
Asp-β-OBz	3	0	84	16	0	67	33
Glu-α-OBz	2	39	43	18	22	41	37
Asp-α-OBz	3	0	90	10	0	80	20
Glu(OEt)$_2$	5	64	36	0	8	40	52
Glu-γ-OEt	6	0	84	16	0	47	53
Asp-β-OEt	6	0	96	4	0	86	14
Gly-OBz	7	—	79	21	—	—	—
Gly-OEt	8	—	88	12	—	—	—

Zwar zeigen die Monoester die gleichen R_F-Werte, führten unsere Versuche dennoch zu dem Ergebnis, dass die Zersetzung der Diester praktisch zu reinen α-Monoestern führt. In der Asparaginsäure-Serie zeigte sich dies in den verschiedenen Farben der beiden Monoester, und in der Glutaminsäure-Serie darin, dass es gelang, aus dem Diester durch Behandlung mit eisessigsaurem HBr das Glu-α-OBz präparativ zu erhalten[9].

Da eine Zersetzung auch bei den Glycinestern beobachtet werden kann, darf man annehmen, dass auf Wirkung von eisessigsaurem HBr jeder Aminosäureester mehr oder weniger zersetzt wird.

EXPERIMENTELLER TEIL

Behandlung mit eisessigsaurem Bromwasserstoff

20 mg des zu untersuchenden Aminosäureesters wurden mit 0.5 ml 30 %igem eisessigsaurem HBr 15 Minuten geschüttelt, die Aminosäure-Derivate dann mit 5 ml Äther gefällt und unter Äther in 5 ml Wasser gelöst. Unmittelbar aus der wässrigen Phase wurde dann die Lösung auf das chromatographische Filterpapier aufgetragen.

Papierchromatographische Untersuchung

Aus der Lösung der zu untersuchenden Probe trugen wir mit Hilfe einer Mikropipette (VEB Glaswerke, Ilmenau) 5–60 γ auf einen Papierstreifen (Schleicher-Schüll 2043a) auf und chromatographierten mit der oberen, organischen Phase eines *n*-Butanol–Eisessig–Wasser (4:1:5) Gemisches 15–16 Stunden mit aufsteigender Technik. Der aus dem Zylinder gehobene Papierstreifen wurde an freier Luft getrocknet, einige Male durch eine 0.2 %ige Ninhydrinlösung (in *n*-Butanol–Chloroform 1:1) gezogen und bei 100 ± 2° genau 10 Minuten getrocknet. Die R_F-Werte der untersuchten Aminosäuren und ihrer Derivate sowie die mit Ninhydrin gebildeten Farben zeigt Tabelle II.

TABELLE II

Aminosäuren und ihre Derivate	*R_F-Wert*	*Farbe*
$Glu(OH)_2$	0.17	violett
$Glu(OBz)_2$	0.82	violett
Glu-γ-OBz	0.58	violett
Glu-α-OBz	0.59	violett
$Glu(OEt)_2$	0.68	violett
Glu-γ-OEt	0.46	violett
$Asp(OH)_2$	0.15	violett
$Asp(OBz)_2$	0.82	gelblich braun
Asp-β-OBz	0.62	gelblich braun
Asp-α-OBz	0.63	violett
$Asp(OEt)_2$	0.65	gelblich braun
Asp-β-OEt	0.35	gelblich braun
Gly-OH	0.12	violett
Gly-OBz	0.65	violett
Gly-OEt	0.43	violett
Gly-OMe	0.27	violett

Densitometrische Auswertung

Die nach der oben beschriebenen Methode entwickelten und gefärbten Chromatogramme wurden mit Hilfe eines JOUAN'schen Densitometers (Ref. 1606) ausgewertet[10]. Der Papierstreifen war nicht lichtdurchlässig präpariert.

Unter Anwendung dieser Technik nahmen wir die Kalibrationskurven mit papierchromatographisch reinen Standardsubstanzen auf (Fig. 1 und 2). Die am Integrator direkt ablesbaren Millimeter-Entfernungen wurden in μg der Aminosäure-Derivate angegeben, und diese Werte in Kenntnis der Molekulargewichte, auf die Mengen der entsprechenden Aminosäuren umgerechnet. Die in der Menge der Grund-Aminosäuren ausgedrückten Werte der einzelnen Flecke wurden aufeinander bezogen und in prozentueller Verteilung angegeben (Tabelle I).

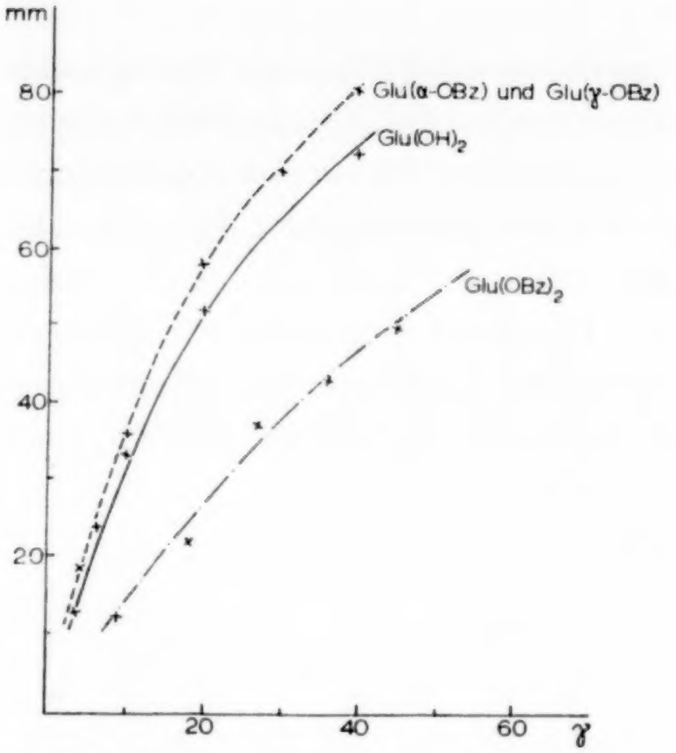

Fig. 1. Kalibrationskurven der Glutaminsäure und ihrer Benzylester.

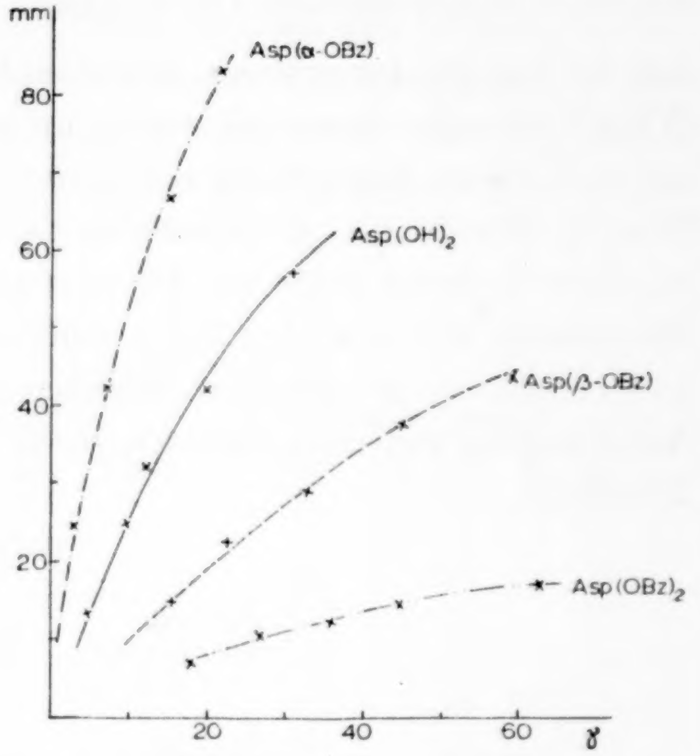

Fig. 2. Kalibrationskurven der Asparaginsäure und ihrer Benzylester.

ZUSAMMENFASSUNG

Es wurde die papierchromatographische Untersuchung einiger Aminosäureester nach Behandlung mit eisessigsaurem Bromwasserstoff durchgeführt. Dabei konnte festgestellt werden, dass diese Behandlung zu einer teilweisen Spaltung der Esterbindungen führt und bei den Peptidsynthesen wird das Hauptprodukt durch diese, in der Nebenreaktion gebildeten, Verbindungen verunreinigt. Das Mass der Zersetzung wurde durch die densitometrische Auswertung der Papierchromatogramme bestimmt.

SUMMARY

Some amino acid esters were investigated by paper chromatography after they had been treated with hydrogen bromide in glacial acetic acid. It was found that this treatment caused partial cleavage of the ester bonds, and that in the synthesis of peptides the main product is contaminated with the compounds formed in this side reaction. The extent of the decomposition was determined by densitometric evaluation of the paper chromatograms.

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THE CHROMATOGRAPHIC BEHAVIOUR OF SOME STRUCTURAL ANALOGUES OF DICARBOXYLIC ACIDS OF THE KREBS CYCLE

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(Received November 14th, 1960)

Much of what is known about the relationships between the biological activity of a compound and its chemical structure has been derived from investigations on structural analogues of metabolic substances. This is the case with studies on enzymic specificity, accumulation of intermediates, inhibitory actions and adaptation processes. When studying the possible biological activity of epoxysuccinic, mercaptosuccinic and mercaptomalic acids, which are structural analogues of oxaloacetic, succinic and malic acids, it became apparent that the chromatographic behaviour of these compounds, in standard conditions, provides a useful means of detecting and separating them.

MATERIALS AND METHODS

Epoxysuccinic, mercaptosuccinic and mercaptomalic* acids were chromatographically pure. Oxaloacetic, succinic, malic, fumaric and tartaric acids, used as reference substances, were all of the best grade available. Mixed solutions of the acids and supernatants of reaction mixtures from biochemical experiments were investigated, and various substances were identified by comparing the R_F values of the unknown spots with those of 1 % standard solutions, using 20 μl per spot.

Ascending chromatography on Macherey-Nagel No. 210 filter paper was employed. Neutral, alkaline and acid solvents were tested; the composition of the solvents, together with the references and development conditions are given in the tables of R_F values. In the experiments with neutral and alkaline solvents, the sodium salts of the acids were also used for comparative tests.

For the detection of the spots, a bromocresol green solution according to PANEK[1], was used as spraying reagent, after the chromatograms had been dried at 50° until constant weight. Higher temperatures were avoided because of the instability of the mercaptoacids. As specific reagents, a 2 % $FeCl_3$ solution was used for the epoxy-, mercapto- and keto-acids, and alkaline silver nitrate for the hydroxyacids.

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* Prepared by Mrs. AURORA GIORA, in the Laboratory of Organic Chemistry of the Faculdade de Filosofia, Ciencias e Letras da Universidade de São Paulo, Brazil.

RESULTS AND DISCUSSION

The R_F values of the free acids in neutral and alkaline solvents were low, although higher than the R_F values of the respective sodium salts, as can be seen in Table I.

TABLE I

R_F VALUES OF DICARBOXYLIC ACIDS IN NEUTRAL AND ALKALINE SOLVENTS

Acids	$R_F \times 100$ *in neutral solvents*						$R_F \times 100$ *in alkaline solvents*				
	A		*B*		*C*		*D*		*E*		*F*
	acid	*salt*	*acid*	*salt*	*acid*	*salt*	*acid*	*salt*	*acid*	*salt*	*acid*
Epoxysuccinic	77;19	10	48;13	08	21;42	06	36	24	59	10	00
Succinic	78	08	15;10	12	06	—	35	28	57	11	09
Mercaptosuccinic	24	09	08	04	14	05	14	11	41	06	00
Mercaptomalic	45;20	21	68;08	04	21	03	23	16	46	06	00
Malic	54	29	68	21	42	—	29	22	53	10	00
Fumaric	49	25	06	04	—	—	42	24	66	13	00

Solvents: A = 95 % ethanol[2]; B = n-butanol–ethanol–water (4:1:1 v/v)[2]; C = phenol–water[2]; D = ethanol–conc. NH_4OH–water (80:5:15 v/v)[3]; E = n-propanol–conc. NH_4OH (70:30 v/v)[1]; F = n-butanol–conc. NH_4OH (90:40 v/v)[4].
Conditions: A, D, E: 7 h, 27°; B, C, F: 18 h, 27°.

"Comets" and double spots were frequent; this had already been observed for citric acid[5]. These inconveniencies disappeared when acid solvents were used, as shown in Table II.

TABLE II

R_F VALUES OF DICARBOXYLIC ACIDS IN ACID SOLVENTS

Acids	$R_F \times 100$					
	G	*H*	*I*	*J*	*K*	*L*
Epoxysuccinic	61	72	77	78	84	61
Succinic	77	79	68	88	88	72
Mercaptosuccinic	85	83	87	88	83	84
Mercaptomalic	83	83	87	90	79	78
Fumaric	91	90	92	92	96	92
Oxaloacetic	29	—	17	—	—	16
Tartaric	30	32	54	63	48	13

Solvents: G = n-butanol–formic acid–water (10:2:5 v/v)[4]; H = butan-2-ol–formic acid–water (85:5:10 v/v)[5]; I = ethyl ether–acetic acid–water (13:3:5 v/v)[4]; J = ethyl acetate–acetic acid–water (2:1:1 v/v)[6]; K = methyl ethyl ketone–acetone–formic acid–water (80:4:2:12 v/v)[7]; L = methyl isobutyl ketone–formic acid–water (40:20:20 v/v).
Conditions: G, H, L: 20 h, 27°; I, J, K: 7 h, 27°.

The best results were obtained with solvents containing formic acid, **Bray** and coworkers[9] referred to similar experiences in the resolution of hydroxyacids. Fumaric acid exhibited the highest R_F value in the solvents tested, as is usual for unsaturated acids while oxaloacetic and tartaric acids had the lowest values. The R_F values of epoxysuccinic acid were in general lower than those of succinic acid, but the R_F values of the mercaptoacids were always higher than those of the respective analogue. These differences are shown in Fig. 1.

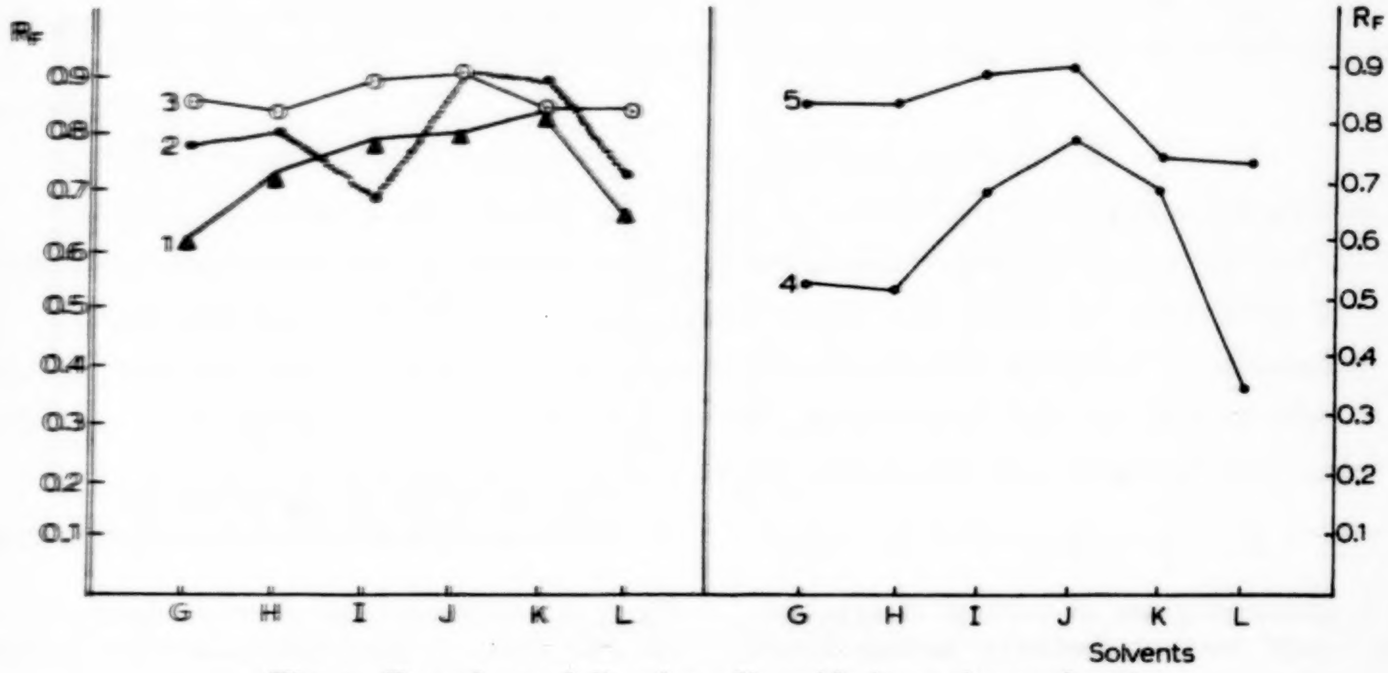
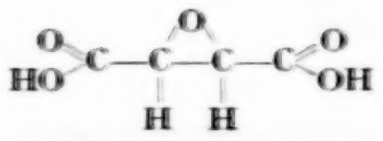
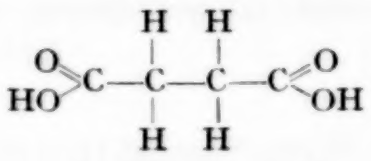


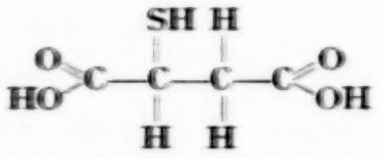
Fig. 1. R_F values of dicarboxylic acids in various solvents.



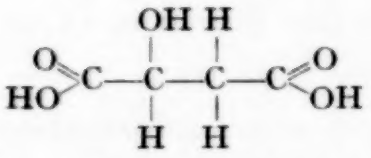
(1) Epoxysuccinic acid



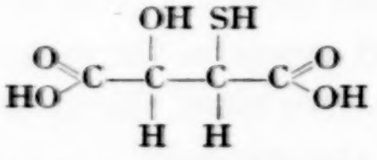
(2) Succinic acid



(3) Mercaptosuccinic acid



(4) Malic acid



(5) Mercaptomalic acid

Solvent L gave the best resolution in all the experiments; a typical chromatogram is reproduced in Fig. 2.

For organic acids REIO[8] used a solvent containing methyl isobutyl ketone and formic acid, but in different proportions and prepared by a more complicated procedure; the R_F values obtained for the same acids are lower in REIO's solvent.

The R_F values of the mercaptoacids were very close together, the acids showing a tendency to ascend together, and good separations could only be achieved by means of two-dimensional chromatography. A pair of solvents was investigated, solvent A (95% ethanol) in the first direction and solvent L (methyl isobutyl ketone–formic acid–water, 40:20:20 v/v) in the second. A typical two-dimensional chromatogram is represented in Fig. 3.

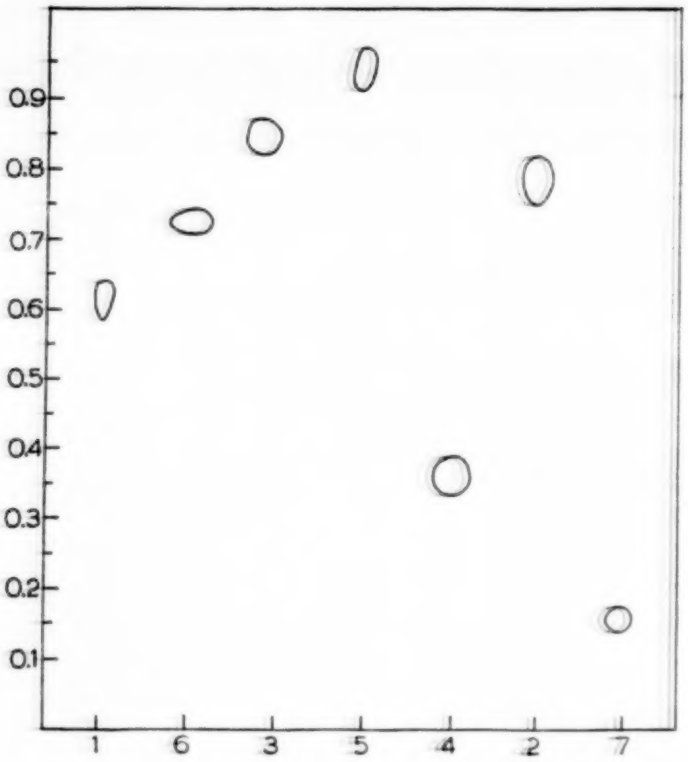


Fig. 2. Chromatogram of various dicarboxylic acids, with methyl isobutyl ketone–formic acid – water (40:20:20 v/v) as solvent. Conditions: 20 h at 27°. (1) Epoxysuccinic acid; (2) mercaptomalic acid; (3) mercaptosuccinic acid; (4) malic acid; (5) fumaric acid; (6) succinic acid; (7) oxaloacetic acid.

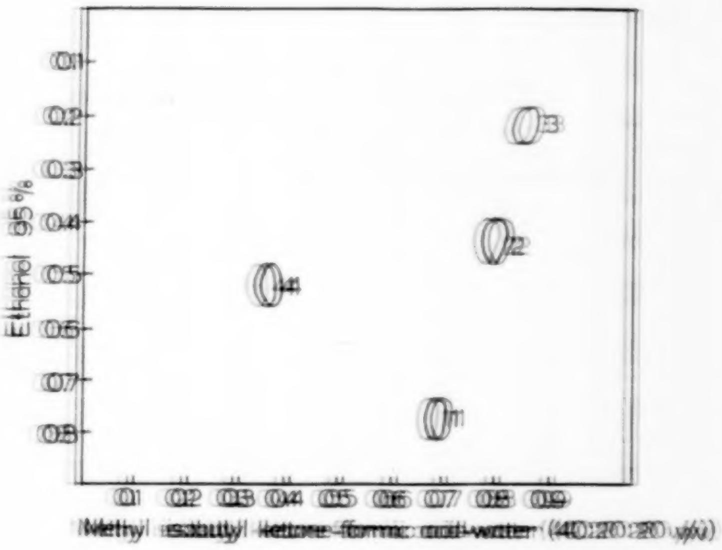


Fig. 3. Two-dimensional chromatogram of: (1) succinic acid; (2) mercaptomalic acid; (3) mercaptosuccinic acid; (4) malic acid.

Recently HOWE[10] found that there is a definite relationship between the R_M value, calculated according to BATE-SMITH AND WESTALL's formula, $R_M = \log (1/R_F - 1)$, and the molecular structure of an acid. By calculating the R_M value, it is possible to obtain some indication of the substituent groups present in a molecule. In an attempt to get some information about the influence of epoxy and sulphydryl groups in the molecule of the various analogues, the R_M values of the acids in the acid solvents used were calculated and these are given in Table III.

The introduction of the SH group in the molecule of the respective analogues lowered the R_M values. On the other hand, the epoxy group raised the R_M values in comparison with the analogous succinic acid.

TABLE III

R_M VALUES OF DICARBOXYLIC ACIDS IN ACID SOLVENTS

Acids	R_M					
	G	*H*	*I*	*J*	*K*	*L*
Epoxysuccinic	−0.19	−0.41	−0.52	−0.55	−0.72	−0.19
Succinic	−0.52	−0.57	−0.33	−0.86	−0.86	−0.41
Mercaptosuccinic	−0.75	−0.79	−0.83	−0.86	−0.79	−0.72
Mercaptomalic	−0.79	−0.79	−0.83	−0.95	−0.57	−0.55
Malic	−0.05	−0.14	−0.35	−0.55	−0.35	+0.27
Fumaric	−1.00	−0.95	−1.06	−1.06	−0.93	−1.06
Oxaloacetic	+0.39	—	+0.69	—	—	+0.72
Tartaric	+0.37	+0.33	−0.07	−0.23	+0.68	+0.83

Oxygenated groups behaved similarly also in the keto-acid and in tartaric acid. This agrees with the observations of HOWE[10] on the OH group, using other solvents.

CONCLUSIONS

1. Acid solvents are more suitable for the separation of the dicarboxylic acids investigated.
2. Solvents containing formic acid gave the best resolutions.
3. Epoxysuccinic acid is easily separated in one-dimensional chromatograms, the most suitable solvent being methyl isobutyl ketone–formic acid–water (40:20:20 v/v).
4. Mercaptosuccinic and mercaptomalic acids can only be separated by two-dimensional chromatography; a procedure was developed using 95% ethanol in one direction and methyl isobutyl ketone–formic acid–water (40:20:20) in the other.
5. In the solvents examined, the SH group raises the R_M value of the respective analogue and the epoxy group lowers it.

ACKNOWLEDGEMENTS

Thanks are due to Dr. HEINRICH HAUPTMANN who encouraged this work; and also to the Conselho de Pesquisas da Universidade do Brasil and Conselho Nacional de Pesquisas for grants.

SUMMARY

A paper chromatographic procedure is described for the separation of epoxysuccinic, mercaptosuccinic and mercaptomalic acids from their structural analogues involved in the Krebs cycle, succinic, fumaric, oxaloacetic and malic acids. Several solvents were assayed and the R_F values recorded. The best critical conditions are discussed and also the influence of the substituent groups on the R_M values of the metabolic compounds.

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ÉTUDE DE L'ISOLEMENT DES CATÉCHOLAMINES URINAIRES AU MOYEN DE L'ALUMINE, EN VUE DE LEUR DOSAGE CHIMIQUE

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(Reçu le 2 décembre 1960)

INTRODUCTION

Le dosage chimique des catécholamines urinaires a donné lieu à de nombreux travaux. Il reste que ces analyses sont considérées, à juste titre, comme extrêmement délicates et peu sûres. Quelle que soit la technique, elle procède invariablement par deux étapes: l'isolement des catécholamines et leur conversion en dérivés dont on mesure la fluorescence. Jusqu'à maintenant on a attaché une importance toute particulière tant aux conditions de formation des dérivés qu'à la manière d'apprécier la fluorescence. Il est cependant remarquable que les données expérimentales sur la première partie du dosage, à savoir l'isolement des catécholamines, soient pratiquement inexistantes, si l'on en excepte les observations de LUND[1] qui remontent déjà à une dizaine d'années. Il s'agit là d'une déficience qui a d'ailleurs été particulièrement signalée au cours d'un symposium récent[2].

Toute technique d'isolement des catécholamines urinaires par chromatographie sur alumine doit répondre aux exigences suivantes: (a) elle doit être reproductible, sinon quantitative; (b) elle doit éliminer toute substance pouvant gêner la détermination fluorimétrique et (c) elle doit éviter l'altération des catécholamines au cours des opérations, en particulier au cours de l'adsorption.

Une étude minutieuse des travaux antérieurs montre que les difficultés rencontrées par leurs auteurs ont leur origine à cette étape et se rattachent à l'une ou l'autre des conditions suivantes:

(a) Écarts parfois considérables dans le rendement. Un travail récent[3], qui rapporte sans commentaires des rendements variant de 15 à 94 %, laisse quelque doute sur la validité de la reproduction.

(b) Élimination des substances fluorescentes de l'urine. Cet obstacle est surmonté si l'on possède un moyen adéquat de mesurer les valeurs du blanc, mais tel ne semble pas toujours le cas[3].

(c) Impuretés provenant de l'alumine elle-même et susceptibles de fausser la détermination fluorimétrique, comme l'a signalé CROUT[4]. De leur côté, PRICE ET PRICE[5] signalent une différence dans les valeurs de fluorescence des catécholamines (par formation des lutines) suivant qu'elles sont dissoutes dans un acide qui a passé ou non sur une colonne d'alumine. Il y a là un indice que l'alumine n'agit pas seulement comme adsorbant.

(d) Enfin, la possibilité d'une transformation partielle des catécholamines par passage sur alumine, a été soulevée par Jones et Blake[6] pour expliquer certaines particularités dont nous allons discuter plus loin.

Malgré ces nombreux indices, une étude systématique du rôle de l'alumine comme agent adsorbant des catécholamines (plus particulièrement de l'adrénaline et de la noradrénaline) faisait jusqu'à maintenant défaut. C'est l'intention du présent travail de parer à cette déficience et de suggérer un mode opératoire qui soit expérimentalement éprouvé.

PARTIE EXPÉRIMENTALE

Matériel et méthode

Réactifs

1. Adrénaline, c.p. (Distillation Products).
2. Bitartrate de noradrénaline, c.p. (Winthrop-Stearns).
3. Bitartrate de β-^{14}C-adrénaline (Tracerlab).
4. Alumines: Brickman Company (Montréal); Woelm ("non alkaline"); Merck ("suitable for chromatographic adsorption"); échantillons L-93-8 et L-93-12 (Aluminium Laboratories Limited, Arvida, Canada).

Dosage des catécholamines

Le dosage des catécholamines a été effectué par l'intermédiaire des lutines, suivant une méthode adaptée de Ehrlén[7] et de von Euler et Floding[8]. La manière d'apprécier les blancs a été légèrement modifiée. Pour les mesures simultanées de l'adrénaline et de la noradrénaline, nous avons adopté la technique d'excitation de la fluorescence par deux longueurs d'onde différentes: un procédé analogue a été employé par Price et Price[5]. La fluorescence a été mesurée avec un fluorimètre construit sur place, utilisant le circuit électronique du spectrophotomètre Beckman, modèle B, et plus tard relié au Microphotomètre Aminco. On a utilisé les filtres suivants:

Filtres primaires: Corning 7-83 isolant la raie de 365 mμ du mercure; Corning 5-74 ou filtre d'interférence (Photovolt) isolant la raie 436 mμ; filtre secondaire: Corning 3-69 (jaune).

Nous avons aussi vérifié que le photofluorimètre Coleman possède une sensibilité suffisante pour ces déterminations.

Choix de la méthode d'adsorption

L'isolement des catécholamines par adsorption et élution successive peut s'effectuer, soit en agitant une suspension d'alumine dans la solution contenant les catécholamines, puis dans une solution éluante (méthode en équilibre), soit en laissant percoler la solution de départ à travers une couche d'alumine immobilisée dans une colonne (chromatographie proprement dite). Le deuxième procédé est infiniment plus efficace pour séparer des substances aux propriétés semblables. Or les séparations que l'on veut obtenir, dans le cas présent, sont plutôt simples d'une part à cause de la grande spécificité de la réaction de formation des lutines et, d'autre part, à cause de la facilité d'évaluer le blanc de fluorescence, comme on le verra plus loin. Dans ces conditions, la méthode d'adsorption et d'élution en équilibre devient le procédé de choix pour les analyses de routine où l'on peut consentir à une faible diminution du rendement au profit d'une manipulation plus simple, plus rapide et offrant les mêmes garanties de précision. C'est le procédé adopté dans la présente étude. Pour des raisons de commodité d'expression et à l'instar des autres auteurs, nous continuerons à l'identifier par le terme de *chromatographie sur alumine*.

Choix de l'alumine

Le choix de l'alumine en vue de la chromatographie mérite une attention certes plus grande qu'on ne lui en a accordée jusqu'à maintenant. La chromatographie consiste en une adsorption des molécules des catécholamines sur la surface même des grains d'alumine, suivie d'une élution. L'efficacité de cette opération dépend donc grandement de l'état physicochimique de la surface (forme cristalline, grandeur de surface active). Comme on peut s'y attendre, les alumines à grande surface donnent des rendements plus élevés. Mais il y a un autre facteur dont il faut tenir compte: c'est l'influence que peut avoir l'alumine passée en solution ou en dispersion colloïdale sur la fluorescence finale des catécholamines. En effet, plus il y a d'alumine en solution, moins la fluorescence spécifique est intense et plus le spectre est déformé, ce qui entraîne de graves erreurs dans les résultats[4,5].

Or il arrive que ce sont, parmi les alumines que nous avons étudiées, celles dont la surface active est la plus grande qui montrent le plus de facilité à passer en solution et donnent les valeurs de fluorescence spécifique[**] les moins élevées. Dans le Tableau I, nous avons réuni ces caractéristiques pour trois alumines commerciales, une alumine traitée sur place (voir description au chapitre suivant) et un échantillon expérimental.

La fluorescence spécifique a été déterminée en ajoutant les deux catécholamines à de l'acide sulfurique 0.2 *N* qui avait été agité auparavant avec chacune des alumines mentionnées (le choix de l'acide est discuté plus loin). On constate que cette fluorescence est la plus faible pour les alumines de Woelm et de Merck, et la plus forte pour celles de Brickman, traitée ou non.

La facilité qu'ont les alumines de passer en solution dans l'acide sulfurique 0.2 *N* a été appréciée en comparant la turbidité produite à pH 8 (minimum de solubilité pour l'oxyde d'alumine) et la diffusion de lumière à pH 8 et à pH 14 (le pH de la mesure fluorimétrique finale). On remarque que ce sont encore les alumines de Merck et de Woelm qui se dissolvent le plus facilement, tandis que celles de Brickman sont les moins solubles.

[**] Au cours du présent travail, on appellera *fluorescence spécifique* la fluorescence de 0.6 µg de noradrénaline et de 0.15 µg d'adrénaline (soit une fraction de 1.55 ml sur 5.0 ml d'éluat) dans un volume final de 7.2 ml. (Voir *Mode opératoire*.)

TABLEAU I

QUELQUES CARACTÉRISTIQUES DES DIVERSES PRÉPARATIONS D'ALUMINE; LEURS INFLUENCES SUR L'EFFICACITÉ DE LA CHROMATOGRAPHIE ET LA MESURE DE LA FLUORESCENCE

Alumine	*Rendement (%)*		*Fluorescence spécifique*[a]				*Blancs de fluorescence*		*Alumine en solution*[b]			*Surface*[e] *interne* *(m²/g)*
	Norad.	*Adr.*	*Noradrénaline*		*Adrénaline*				*Turbidité*[c]	*Diffusion*[d]		
			365 mμ	*436 mμ*	*365 mμ*	*436 mμ*	*365 mμ*	*436 mμ*		*pH 8*	*pH 11*	
Brickman	69	41	51.5	32	19	46.5	7	16	6.5	8	20	9.1
Brickman lavée	84	34	53.5	37	20.5	47	6	13.5	4.0	2.5	15	10.9
L-93-8	105	50	49.5	34	18.5	43	7.5	16	12.0	24	16.5	23.1
Merck	86	47	35.5	25.5	15	35.5	17	24	17.0	48	12.5	—
Woelm	85	74	40	28	20	46	15	23	39.0	100	50	91.5
(Éluant pur)			58.5	37	22.5	50	6.5	15				

[a] Les unités sont arbitraires; une solution de référence (fluorescéine à 10 μg/l, tamponnée à pH 8.2) correspond à 15 unités à 365 mμ et à 80 unités à 436 mμ. Les valeurs sont corrigées pour celles des blancs.

[b] Les mesures de la turbidité et de la diffusion de la lumière sont des valeurs moyennes; les autres chiffres sont des valeurs typiques d'une seule détermination.

[c] La turbidité fut mesurée au Photrometer Rouy-Leitz en pourcentage d'absorption.

[d] La diffusion lumineuse fut mesurée au fluorimètre avec deux filtres, primaire et secondaire, de même longueur d'onde (436 mμ). Les unités sont arbitraires.

[e] Mesurée par adsorption d'azote. Ref.[10].

On remarque par ailleurs que les alumines les plus solubles (L-93-8, Woelm) possèdent les surfaces actives les plus grandes et donnent, en partant de solutions pures des catécholamines, un meilleur rendement chromatographique.

On est donc amené à choisir entre les alumines à bon rendement, à fluorescence spécifique faible avec spectre déformé et les alumines à rendement plus faible mais permettant de développer une fluorescence se rapprochant de celles des solutions pures.

Nous avons opté pour les secondes, leur faible solubilité offrant les meilleures garanties de reproduction, par contraste avec les alumines du genre "solubles" dont la reproduction est moins sûre.

Traitement de l'alumine

Pour nous assurer une qualité plus uniforme de l'alumine, nous avons mis au point un procédé de lavage et de séchage. En fait, on doit considérer cette opération comme un traitement physico-chimique beaucoup plus qu'un simple lavage. L'alumine est traitée par l'acide chlorhydrique azéotropique dans un extracteur Soxhlet pendant au moins une journée entière, puis rincée à plusieurs reprises avec de l'eau distillée. L'extraction au Soxhlet est ensuite reprise avec de l'acide acétique glacial pour une autre journée. Suit alors une longue série de lavages à l'eau distillée jusqu'à ce que l'eau de lavage, trouble au début, devienne parfaitement limpide. On sèche alors à 400° pour deux à trois heures. Un séchage plus court ou plus prolongé diminue l'activité de l'alumine et conséquemment le rendement d'adsorption, comme l'illustrent les quelques données du Tableau II.

Il est intéressant de noter que l'acide chlorhydrique ayant servi à l'extraction de l'alumine se colore fortement en jaune et renferme (comme d'ailleurs aussi l'acide acétique de la seconde extraction) un fort résidu solide. Or on sait que les diverses formes d'alumine calcinée sont insolubles dans ces acides. On peut donc en conclure que ces résidus proviennent principalement d'impuretés et très probablement d'alumine hydratée ou carbonatée.

Dans le but de se mettre à l'abri des variations dues à des lots différents, il est suggéré d'en étudier la solubilité (par des mesures de turbidité et de dispersion lumineuse) dans les conditions opératoires et de procéder à un traitement, le cas échéant.

TABLEAU II

INFLUENCE DU TEMPS DE SÉCHAGE DE L'ALUMINE (à 400°) SUR LA RÉCUPÉRATION DE L'ADRÉNALINE ET DE LA NORADRÉNALINE*

Durée du séchage (heures)	*Pourcentage de récupération*	
	*Adrénaline***	*Noradrénaline***
0	0	0
1	19	24
2	31	61
3	31	57
20	40	38

* Dans cette expérience, comme dans les suivantes, l'alumine utilisée fut celle de Brickman (à moins qu'on ne le spécifie autrement).

** En solution dans 20 ml d'urine et 20 ml d'eau.

Chromatographie de solutions aqueuses de mélanges de catécholamines

Dans le cas qui nous intéresse, le résultat final de la séparation chromatographique dépend, en plus de l'état physicochimique de l'adsorbant, des facteurs suivants: (a) de l'état d'ionisation de la molécule des catécholamines, (b) du rapport des volumes des deux phases en présence (ou, plus exactement, du rapport entre le volume de la phase liquide et la surface active du solide), (c) de la nature chimique de l'éluat et enfin (d) de la présence d'autres substances pouvant entrer en compétition avec les catécholamines. Pour mieux mettre en évidence l'effect des trois premiers facteurs (a), (b) et (c), nous avons d'abord procédé à l'étude de solutions des catécholamines dans l'eau pure. Le dernier facteur sera étudié au chapitre de la chromatographie des urines.

(*a*) *État de la molécule des catécholamines*. L'adrénaline et la noradrénaline sont des molécules amphotères dont la charge électrique dépend du pH du milieu. On sait qu'en milieu alcalin, la molécule possède une charge négative et est fortement adsorbée sur l'alumine, alors qu'en milieu acide le renversement de la polarité lui fait perdre toute affinité pour l'adsorbant. Dans ce domaine, nous n'avons fait que confirmer le bien-fondé des conditions adoptées par les premiers chercheurs[1]: l'adsorption est à son maximum à pH 8-9, diminue rapidement avec le pH pour devenir négligeable en solution acide.

(*b*) *Influence du volume de la phase liquide*. L'efficacité de l'adsorption, en outre du pH d'équilibre, dépend grandement du volume initial de la solution utilisée (pour une quantité donnée d'alumine). C'est un facteur dont on ne semble pas avoir tenu suffisamment compte jusqu'à maintenant. En effet, plus le volume de la solution des catécholamines est grand, plus la fraction non adsorbée est abondante. La Fig. 1 rend compte de l'ordre de grandeur de cet effet. Les conclusions de cette expérience sont évidentes: (a) il y a avantage à prendre des volumes faibles et (b) il est nécessaire de garder le volume constant.

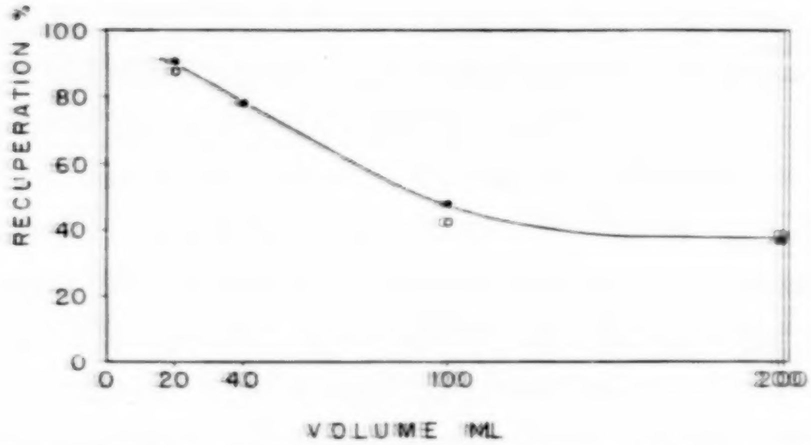

Fig. 1. Influence du volume de la phase liquide sur l'efficacité de l'adsorption. ○, Noradrénaline, ●, Adrénaline.

(*c*) *Choix de l'éluant*. Dans le choix de l'éluant, on doit se guider non seulement sur sa faculté de faire passer, de l'alumine à la solution, les catécholamines, mais aussi sur l'influence qu'il exerce sur la fluorescence finale. Cette influence se fait sentir à deux niveaux: sur le rendement dans la formation des lutines (c'est le cas de l'acide oxalique) et aussi sur la mesure même de la fluorescence à cause de son pouvoir de mise en solution de l'alumine.

Les solutions éluantes que nous avons étudiées nous ont été inspirées par les chercheurs avant nous: solutions d'acide sulfurique 0.2 *N*, d'acide acétique 0.2 *N* et d'acide oxalique *N*.

La première particularité que l'on observe est la suivante: si l'on forme les lutines en partant de catécholamines dissoutes dans des solutions pures des acides mentionnés, on constate que l'intensité de la fluorescence, de même ordre de grandeur pour les acides acétique et sulfurique, est de beaucoup plus basse pour l'acide oxalique (Tableau III). Dans le but de corriger cet inconvénient, Pekkarinen et Pitkänen[9] suggèrent de diluer l'éluat dix fois avant d'effectuer la réaction. Ce procédé nous a paru peu intéressant car il diminue la concentration des catécholamines tout en augmentant la proportion relative des réactifs et conséquemment des blancs. Pour cette raison, l'étude de l'acide oxalique ne fut pas poussée plus loin.

On constate aussi que la fluorescence spécifique développée après agitation de l'éluat avec l'alumine est plus faible que dans l'éluat pur, l'abaissement est plus marqué pour l'acide sulfurique que pour l'acide acétique. Cependant pour une raison que nous n'avons pas pu élucider, la reproduction est nettement meilleure avec l'acide sulfurique (coefficient de variation pour 10 échantillons, $v = 2.0\,\%$) qu'avec l'acide acétique ($v = 6.5\,\%$). C'est pour cette raison que notre choix final s'est porté sur l'acide sulfurique.

TABLEAU III

FLUORESCENCE DE L'ADRÉNALINE ET DE LA NORADRÉNALINE DANS DIVERS ÉLUANTS, PURS OU AGITÉS AVEC L'ALUMINE

Catécholamine en solution dans le solvant pur	*Traitement subséquent*	*Intensité de la fluorescence**					
		Acide sulfurique 0.2 N		*Acide acétique 0.2 N*		*Acide oxalique N*	
		365 mμ	*436 mμ*	*365 mμ*	*436 mμ*	*365 mμ*	*436 mμ*
Noradrénaline	—	83.5	46.5	73	41.5	30.5	20.5
Noradrénaline	agitée avec alumine	50	39.5	65	40.5	24.5	16
Adrénaline	—	27.5	63	24	62.5	5.5	37
Adrénaline	agitée avec alumine	15.5	54	20	53	4.5	34

* Unités arbitraires. Les valeurs sont corrigées pour les blancs. Solution de référence: voir Tableau I, note[a].

Chromatographie des catécholamines dans les urines

Si l'on fait les expériences précédentes sur des urines placées dans les mêmes conditions que les solutions aqueuses, on note des différences très nettes. D'abord la récupération de catécholamines ajoutées à l'urine est définitivement inférieure; elle varie considérablement d'une urine à l'autre; enfin les écarts entre deux déterminations sur un même échantillon sont plus considérables.

C'est particulièrement le manque de fidélité dans la reproduction qui nous a paru le plus important à élucider. Le pH d'adsorption joue ici un rôle encore plus critique que dans les solutions aqueuses: non seulement des variations de l'ordre du dixième d'unité se traduisent par des écarts notables dans le résultat final, mais encore les tâtonnements eux-mêmes de l'ajustement peuvent lui être funestes. PRICE ET PRICE[5] d'ailleurs rejettent systématiquement les échantillons dont le pH a dépassé accidentellement la valeur désirée. Cet effet est plus critique pour certaines urines que pour d'autres: nous avons pu finalement le relier à leur teneur plus ou moins grande en phosphates. Apparemment l'alcalinisation des urines entraînerait une précipitation des phosphates à des pH légèrement variables pour des urines différentes, précipitation non complètement réversible au cours des réajustements de pH dans les intervalles considérés (*i.e.* de 8 à 9). Ce phénomène se répercuterait sur la fluorescence finale, soit par entraînement d'une partie des catécholamines par précipité de phosphates, soit par transfert des phosphates eux-mêmes jusqu'à l'éluat et reprécipitation dans le milieu final fortement alcalin.

Ce rôle des phosphates a été mis en évidence au cours d'une expérience où nous avons comparé l'efficacité de l'étape chromatographique sur les solutions suivantes:

(1) Catécholamines ajoutées à de l'eau pure.

(2) Catécholamines ajoutées à une suspension de phosphate ammoniaco-magné-

sien (obtenu par précipitation d'urines avec l'ammoniaque et lavage du précipité avec de l'eau ammoniacale).

(3) Catécholamines ajoutées à une suspension identique de phosphate et d'un agent chélant (EDTA). La quantité de EDTA était juste suffisante pour complexer l'ion Mg^{++} et empêcher ainsi la reprécipitation des phosphates à pH 8. Nous avons obtenu des rendements suivants: pour la noradrénaline 63.0% et 67.5% dans de l'eau pure; 16.0% et 24.0% dans la suspension de phosphate et de 36.3% et 43.7% dans le phosphate additionné de EDTA. (Les chiffres correspondants pour l'adrénaline sont: 44%–36%; 12.8%–19.4% et 15.2%–14.6%.)

On voit d'abord que la présence de phosphate, dissous ou non, abaisse la fluorescence finale moyenne. Cependant on remarque aussi que le reproduction, plutôt mauvaise en présence de phosphate, est améliorée par l'emploi du EDTA. Dans la pratique des dosages de routine, nous avons trouvé commode d'ajouter, sous forme solide, environ 100 mg de EDTA sodique aux 20 ml d'urine requis dans le mode opératoire suggéré plus loin. Exceptionnellement on est amené à en ajouter une petite quantité supplémentaire lorsque les urines affichent un trouble persistant.

Comme moyen d'améliorer le rendement de la chromatographie des urines, on peut invoquer l'avantage d'une dilution préalable de l'échantillon. Pour vérifier cette possibilité, nous avons ajouté à diverses dilutions d'une même urine des quantités connues, mais faibles des deux catécholamines (0.5 μg d'adrénaline et 2.0 μg de noradrénaline par 20 ml d'urine), quantités qui ne font que doubler approximativement les teneurs normales et ne doivent pas affecter ainsi l'efficacité de l'opération. Cette précaution est nécessaire si l'on veut reporter le résultat d'une telle expérience à l'échelle des catécholamines endogènes[2]. Le Tableau IV illustre le résultat d'une expérience typique où l'on voit que la dilution, en plus d'améliorer le rendement de la chromatographie, permet une meilleure élimination des substances fluorescentes des urines, comme le montrent les valeurs des blancs. (Nous croyons aussi que les écarts de rendement d'une urine à l'autre pourraient s'expliquer par la dilution variable des substances interférentes dans l'échantillon original.)

TABLEAU IV

EFFET DE LA DILUTION D'URINE SUR LA RÉCUPÉRATION D'ADRÉNALINE ET DE NORADRÉNALINE AJOUTÉES*

Quantité (ml)		*Pourcentage de rendement*		*Fluorescence des blancs***	
d'urine	*d'eau*	*Adrénaline*	*Noradrénaline*	*365 mμ*	*436 mμ*
40	0	15.3	19.2	41.5	53.5
20	20	46.3	48.2	20.5	46.0
10	30	64.6	66.0	9.5	16.0
0	40	100.0***	100.0***	5.0	12.5

* Résultats d'une expérience typique sur une urine donnée.
** Unités arbitraires.
*** Calculé comme 100%.

Dans cette expérience, le volume de la phase liquide à chromatographier était de 40 ml dans chaque cas. Si, par contre, l'on augmente le volume initial en portant l'échantillon à 200 ml, il y a une diminution considérable du rendement (comme on l'a d'ailleurs vu précédemment dans le cas des solutions dans l'eau). Ainsi, dans un essai typique, la récupération des catécholamines ajoutées est tombée de 25 à 3% quand nous avons pris un volume initial d'urine de 200 ml (au lieu de 40 ml).

Donc, à toute fin pratique, dans le choix des conditions les meilleures, il faut retenir que (a) le volume de la phase liquide doit être faible, sinon le rendement diminue, (b) la quantité des urines doit être suffisante, de sorte qu'il existe une différence appréciable entre la fluorescence due aux catécholamines et celles de blancs de réactifs, (c) la dilution doit être la plus grande qui soit compatible avec les deux conditions précédentes.

En compromis, nous avons opté pour les conditions suivantes: un volume initial d'urine de 10 ml dilué avec 30 ml d'eau distillée, soit un volume total de 40 ml chromatographié en présence d'un gramme d'alumine. Dans ces conditions, les rendements moyens pour un grand nombre de déterminations sont de 43.5% pour la noradrénaline et de 46.0% pour l'adrénaline. Ce sont ces pourcentages que nous avons utilisés pour établir notre équation générale (voir plus loin).

Suggestion d'un mode opératoire pour l'isolement des catécholamines au moyen de l'alumine

À la suite de l'étude décrite dans la partie expérimentale, nous suggérons le mode opératoire suivant comme offrant le plus de commodité et les meilleures garanties de précision.

(*a*) *Réactifs.* Solution standard d'adrénaline: Solution à 10 mg/l, acidulée; diluer 10 fois avec de l'eau acidulée avant usage (0.5 ml contient 0.5 μg).

Solution standard de noradrénaline: Solution à 10 mg/l acidulée (0.2 ml contient 2.0 μg).

Alumine.

Sel disodique de l'acide éthylènediaminetétraacétique (EDTA).

Phénolphthaleine à 1% (solution alcoolique).

NaOH 0.1 *N*.

H_2SO_4 0.2 *N*.

$NaHCO_3$ à 2%.

Tampon à l'acétate, pH 6: Préparer une solution d'acétate de sodium à 2%; ajuster à pH 6 avec de l'acide acétique.

Ferricyanure de potassium à 0.25%.

Mélange NaOH–acide ascorbique (préparer immédiatement avant usage): Peser 0.2 g d'acide ascorbique et porter à 10 ml; prendre 1 ml et mélanger à 9 ml de NaOH 5 *N*.

(*b*) *Chromatographie.* On recueille les urines des 24 h sur 5 ml d'acide sulfurique concentré. À l'arrivée au laboratoire, on ajuste le pH entre 2 et 3 avec de l'acide sulfurique et on mesure le volume total. On prélève un échantillon de 20 ml, on dissout environ 100 mg de EDTA sodique et on divise en deux portions, l'une servant de standard "interne", et l'autre d'inconnu. À la première on ajoute 0.5 μg d'adrénaline et 2.0 μg de noradrénaline. À partir de ce moment, les deux échantillons sont traités de la même manière. On leur ajoute 30 ml d'eau distillée, 1 g d'alumine, quelques gouttes de phénolphthaléine à 1 % et on ajuste le pH à 8.5 au moyen d'une solution de NaOH 0.1 *N* (la soude est ajoutée jusqu'à la teinte rose pâle, puis on ajuste avec un pH-mètre). L'adsorption se fait par agitation durant quatre minutes. On laisse déposer l'alumine et on décante le liquide surnageant. On fait suivre quatre lavages avec 20 ml d'eau distillée. On centrifuge brièvement, on décante et on assèche les parois avec une tige montée d'ouate ou une pointe de papier filtre. L'élution se fait ensuite par agitation avec 5 ml d'acide sulfurique 0.2 *N* pendant quatre minutes, suivie d'une brève centrifugation. On prélève ensuite deux portions de 1.5 ml de l'éluat du standard et deux portions de 1.5 ml de celui de l'inconnu: elles serviront à déterminer respectivement la fluorescence totale et celle des blancs des réactifs.

Formation des lutines

A chacun des quatre échantillons, on ajoute 1.5 ml de $NaHCO_3$ à 2 %, 3 ml du tampon à l'acétate (pH 6). A un standard et à un inconnu, on ajoute 0.2 ml de ferricyanure à 0.25 %, on mélange et on laisse reposer deux minutes. On ajoute 1 ml du mélange NaOH–acide ascorbique. Pour les deux blancs restant, on inverse l'ordre de ces deux derniers réactifs, soit le mélange NaOH–acide ascorbique suivi du ferricyanure. On mesure pour les quatre échantillons la fluorescence produite par les deux sources excitatrices (raies 365 mμ et 435 mμ du mercure) par rapport à une solution de référence (une solution fraîche de 10 μg au litre de fluorescéine tamponnée à pH 8.2 ou, mieux, un standard plus stable, comme un verre fluorescent). On obtient donc en définitive huit mesures pour chaque urine:

un standard et un blanc à 365 et à 436 mμ;
un inconnu et un blanc à 365 et à 436 mμ.

Dans toutes nos déterminations, le blanc de l'inconnu et le blanc du standard se sont avérés identiques, ce qui justifie notre manière de mesurer le blanc et confirme l'uniformité des préparations d'alumine. Les calculs peuvent donc se simplifier comme suit: (a) La différence des lectures du standard et de l'inconnu est due aux *catécholamines ajoutées*. Elles permettent d'apprécier le rendement total de l'opération. (b) La différence des lectures de l'inconnu et du blanc est due aux *catécholamines endogènes*. Ces valeurs sont corrigées pour le rendement trouvé en (a).

Équations générales

Les équations générales servant à établir la teneur en adrénaline (*A*) et en noradrénaline (*N*) sont obtenues en faisant la moyenne d'un nombre suffisant de déterminations sur des urines additionnées de quantités connues des deux catécholamines et soumises au mode opératoire suggéré. On obtient des équations du genre suivant (ces équations ne sont offertes qu'à titre d'exemple, les coefficients variant évidemment avec l'appareil, les filtres et la solution de référence);

F = unités arbitraires de fluorescence

$$A = \frac{8\,F_{436} - 5\,F_{365}}{356}$$

$$N = \frac{5\,F_{365} - 2\,F_{436}}{58.5}$$

(lectures de la solution de référence: 15 unités à 365 mμ et 80 à 435 mμ).

On peut, après un certain nombre d'analyses, refaire la moyenne des valeurs obtenues, et corriger les coefficients des équations générales pour les écarts constatés entre les quantités de catécholamines ajoutées et la moyenne des quantités retrouvées. Toutefois, cette précaution n'est pas essentielle puisque la présence des standards "internes" assure la correction.

Considérations additionnelles sur l'emploi de l'alumine comme agent adsorbant

Bien que les résultats ci-haut mentionnés aient permis d'élaborer un mode opératoire satisfaisant en pratique, il restait encore des aspects non élucidés dans l'emploi de l'alumine comme agent adsorbant. Certaines observations décrites dans la littérature ont suggéré que l'alumine, soit sous sa forme solide, soit même à l'état dissous, joue le rôle, non seulement d'un adsorbant inerte, mais d'un agent chimique (ou catalytique) pouvant altérer les catécholamines. En effet, divers auteurs ont noté certaines anomalies apparentes que nous allons énumérer ici.

(1) Ainsi, la fluorescence des lutines est différente selon qu'elles sont formées dans un éluant acide qui a été ou non en contact avec l'alumine. Cette différence serait d'ailleurs non seulement quantitative, mais aussi qualitative: le rapport entre les fluorescences excitées par 365 mμ et 436 mμ n'est pas le même. Le phénomène est rapporté par PRICE ET PRICE[5] qui, dans la préparation des standards, spécifient que "[the] addition [of catecholamines] made to reagents which have not been exposed to alumina are inacceptable for analytic purposes because the fluorescence observed per microgram of E and NE and ratios of fluorescence observed using the 400 and 436 primary filters are modified by the presence of the acetic acid eluate of alumina". Les auteurs, cependant, n'offrent aucune tentative d'explication de ce phénomène.

(2) CROUT[4], de son côté, explique le changement dans le niveau de la fluorescence totale (sans tenir compte des sources différentes de lumière excitatrice) par un effet de simple extinction ("quenching") due à la perte de lumière excitatrice par dispersion sur des microscristaux d'alumine en suspension.

(3) Enfin, JONES ET BLAKE[6] signalent l'anomalie suivante: quand une solution de noradrénaline, après avoir été soumise à une première chromatographie, est ramenée aux conditions initiales de volume et de pH et est rechromatographiée, le rendement de la seconde opération n'est que de 50% de celui de la première. Les auteurs suggèrent explicitement une transformation d'une partie de la catécholamine au cours du premier contact avec l'alumine, fraction pour laquelle l'adsorbant n'aurait plus la même affinité.

Nous avons repris cette étude dans l'espoir d'y apporter quelques lumières.

(a) L'influence de l'alumine sur la fluorescence (des lutines) obtenue par excitation à diverses longueurs d'onde est illustrée au Tableau I. On y voit que le contact de l'alumine change le rapport des fluorescences obtenues par des excitations différentes, comme l'avaient signalé PRICE ET PRICE. Cependant les écarts sont relativement faibles et pourraient s'expliquer par un autre mécanisme que celui de la formation de dérivés de nature différente.

Pour confirmer l'action possible de l'alumine sur les mesures de fluorescence elle-même, nous avons ajouté des aliquotes d'une solution de fluorescéine à l'éluant acide qui avait été auparavant mis au contact de diverses alumines. Le pH des solutions fut ensuite ramené à 8 et la fluorescence mesurée pour les deux raies d'exitation mentionnées. Le Tableau V montre que les fluorescences spécifiques sont changées et que le rapport des fluorescences selon les sources d'exitation n'est plus le même d'une alumine à l'autre. On remarquera que ce rapport augmente dans le même ordre que la solubilité des alumines (il faut souligner que l'effect est ici exagéré par la grande insolubilité de l'alumine au pH employé (soit 8) à contraster avec le pH final dans le cas des catécholamines (soit 14).

TABLEAU V

INFLUENCE DE DIVERSES ALUMINES SUR LA FLUORESCENCE SPÉCIFIQUE DE LA FLUORESCÉINE À pH 8

Alumine utilisée	*Fluorescence (unités art.)*		*Rapport de la fluorescence*	*Turbidité à pH 8*
	365 mμ	*436 mμ*	*436/365 mμ*	
Fluorescéine seule	15.0	16.0	1.07	—
L-93-12	22.0	27.0	1.23	3
Brickman	25.0	31.5	1.26	6
L-93-8	29.5	38.0	1.29	11
Woelm	57.0	97.0	1.70	28

(b) L'influence que peut avoir une première adsorption sur le comportement des catécholamines lors d'une seconde adsorption sur l'alumine, a été étudiée grâce à l'adrénaline marquée (^{14}C). Les mesures ont été effectuées à la fois par fluorimétrie et par mesure de la radioactivité, et les résultats obtenus furent les suivants: rendement de la 1ère chromatographie: 80 % par la fluorescence et 81 % par la radioactivité; rendement de la 2ième chromatographie: 59 % par la fluorescence et 56 % par la radioactivité. L'accord entre les deux résultats indique qu'il ne s'agit pas d'un abaissement de fluorescence par des substances étrangères, mais bien d'une diminution du matériel récupéré. Le Tableau VI montre qu'il y a effectivement une baisse de rendement considérable pour la plupart des alumines et aussi que cette perte se fait sentir surtout pour les alumines les plus solubles (caractérisées par une forte turbidité et une grande surface interne). L'explication du phénomène est en réalité fort simple: l'éluat acide au pH d'adsorption (8.5) laisse précipiter l'alumine dissoute sous forme

TABLEAU VI

RENDEMENT DES CHROMATOGRAPHIES SUCCESSIVES DE LA NORADRÉNALINE SUR DIVERSES ALUMINES

Alumine	*Rendement (%)**		*Rapport de l'efficacité de la 2ième à la 1ère*	*Surface interne (m^2/g)*	*Turbidité à pH 8*
	1ère chromatographie	*2ième chromatographie*			
L-93-12	53.0 (53.5)	51.5 (74.0)	0.97 (1.38)	2.1	3
Brickman	50.5 (52.5)	27.0 (17.1)	0.54 (0.33)	9.1	6
L-93-8	79.0 (84.0)	45.0 (64.0)	0.57 (0.76)	23.1	11
Woelm	89.0 (100.0)	39.0 (29.0)	0.44 (0.29)	91.5	28

* Les rendements sont calculés sur la base d'une seule mesure de fluorescence avec λ excitation : 365 mμ ; les chiffres entre parenthèses sont ceux calculés avec λ excitation : 436 mμ pour les mêmes échantillons.

d'un gel très fin (visible toutefois à l'oeil nu pour les alumines les plus solubles telles que celle de Woelm). Ce gel adsorbe une partie des catécholamines et est emporté facilement au cours des décantations et des lavages, par contraste avec l'alumine franchement cristalline qui sédimente facilement.

À notre avis, ces diverses expériences fournissent l'explication des anomalies apparentes qui ont été à la base de l'hypothèse d'une altération chimique des catécholamines.

REMERCIEMENTS

Les auteurs tiennent à remercier le docteur J. G. LINDSAY, Ph. D., (Aluminium Laboratories Limited, Arvida) qui nous a fourni les échantillons expérimentaux d'alumine et a effectué les déterminations physico-chimiques sur les diverses alumines utilisées au cours du présent travail; mesdemoiselles LOUISE VACHON, d.t.m., et MADELEINE LIZOTTE, R. T. (Bioch.) pour leur précieuse collaboration technique.

RÉSUMÉ

À la lumière des propriétés physico-chimiques de diverses préparations commerciales et expérimentales d'alumines, il a été possible d'élucider les conditions d'adsorption de l'adrénaline et de la noradrénaline, en vue de leur dosage chimique dans l'urine. Cette étude a permis d'élaborer un mode opératoire fiable qui, entre autres, fait usage d'un double standard "interne" pour tenir compte du comportement différent des deux catécholamines au cours de l'adsorption et au cours de la formation des lutines fluorescentes.

Enfin des expériences additionnelles ont servi à mettre sérieusement en doute l'hypothèse d'une altération chimique des catécholamines au contact de l'alumine, comme le suggéraient diverses observations d'autres chercheurs.

SUMMARY

The physicochemical characteristics of various commercial and experimental aluminas have been investigated: those with greater internal surface areas are more efficient

as adsorbents, but they are also more soluble during acid elution. This affects the final fluorescence of lutins qualitatively (spectrum distortion) as well as quantitatively. Although some types of alumina appear to be usable as such for routine analysis, a procedure is suggested for obtaining a uniformly reliable material.

Adsorption of catecholamines is maximal at pH 8-9. Its efficiency is decreased by increasing the volume of the liquid phase. Acid eluents differ in their behaviour; they have a direct influence on the fluorescence of lutins, and an indirect one dependent upon their solvent action on alumina. Sulphuric acid (0.2 *N*) is recommended as most suitable.

When the above-mentioned criteria are applied to urine, much lower yields and poorer reproducibility are encountered. One difficulty, probably due to poorly soluble phosphates, is overcome by using a chelating agent. Dilution of the urine prior to adsorption reduces the concentration of interfering material but adversely affects adsorption: optimum conditions are obtained by diluting 10 ml of sample with 30 ml of water per gram of alumina.

Since these factors do not affect adrenaline (A) and noradrenaline (N) to the same degree, a detailed procedure is described which makes use of both catecholamines as "internal" standards in amounts approximating those found in biological samples (0.5 μg of A; 2.0 μg of N per 10 ml of urine). These standards also provide for the different behaviour of A and N during lutin formation. Blanks of both "internal" standards and unknowns are identical when the oxidizing agent (ferricyanide) and the stabilizer (ascorbate) are reversed in order, an argument in favour of the reliability of such a procedure. Differential estimation is performed by calculating the respective contribution of A and of N to the fluorescence developed under two different activation sources, as suggested by PRICE AND PRICE.

Previous observations by some workers had suggested that catecholamines might be chemically altered by contact with alumina. Evidence is offered that such apparent anomalies are due to a purely mechanical loss of catecholamines during manipulations, especially when "soluble-type" aluminas are used.

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Short Communications

Gas–liquid chromatography: The introduction of samples, the preconditioning of polyester liquid phases and the measurement of R_F values in the analysis of fatty esters

The purpose of this communication is to describe some improvements in the operation of gas–liquid chromatography relating to preconditioning of polyester columns, the ease and accuracy of sample introduction, the consequences of detector overloading and the accurate measurement of R_F values. Some hitherto unpublished R_F values of methyl esters of fatty acids are also recorded.

The gas–liquid chromatograms used employ an ionising detector based on that of LOVELOCK, JAMES AND PIPER[1]. The columns were 254 cm × 6 mm o.d. 35 to 80 mesh "Celite 545" was used as solid support and argon as carrier gas, which was led into the top of the column via a B10 Quickfit joint.

Preconditioning of polyester columns

Reported methods for preconditioning of polyester columns appear to be confined to preheating of the column or the treatment of the polyester with ion exchange resins. In our experience neither method proved completely satisfactory. As an alternative it has been found better to heat the polyester to 260° and pass nitrogen through it for several days until the volatiles are completely removed. The residual polyester became dark and very viscous and was ready for immediate use at 200°.

Sample introduction

Most gas–liquid chromatograms use either micro-pipettes or an injection system for the introduction of non-gaseous samples. However, with both systems the size of samples is difficult to determine accurately. A system was therefore devised whereby the sample is introduced into the column on the inside of a short sampling tube (10 × 5 mm i.d.), which is open at both ends, with a ground inner surface to facilitate spreading of the sample and thus its rapid evaporation. The sample is introduced into the sampling tube with an "Agla" micro-syringe with square tip or preferentially with a fine glass capillary, in which case the relative amounts of sample can be determined by measuring the length of the liquid in the capillary. For quantitative work the sample is weighed and the areas compared with those of a weighed standard such as methyl stearate.

To introduce the sampling tube the argon is turned off and the tube dropped into the top of the column after half a minute when the backpressure in the latter is eliminated. The flow of argon is then resumed. The sampling tubes are recovered from the column at the end of the day with a piece of wire bent slightly at one end.

With this method of sample introduction reproducibility of peak areas, as determined with samples of methyl laurate varying from 40 to 140 μg, was 9.45 area units per 10μ g with a standard deviation S.D. = 0.52 units.

Detector overloading

The effects of overloading of ionising detectors have been described by FARQUHAR *et al.*[2]. This results in blunted or inverted peaks and is particularly apt to occur when low amplification and detector voltages are used with large samples containing relatively volatile esters which issue from the column in narrow bands of high concentration. The difficulty can be overcome by using detector voltages of 1,000 V or more, so that faulty peaks due to overloading exceed the height of the chart and are thereby excluded from normal quantitative treatment.

Table I shows the effect of varying degrees of overloading on the composition of an equimolar six-component mixture of methyl esters. These are denoted as by FARQUHAR *et al.*[2]. The table shows the percent recovery of each ester as determined by the peak areas.

TABLE I

THE EFFECT OF DETECTOR OVERLOADING ON THE COMPOSITION OF AN EQUIMOLAR MIXTURE

Overloading	*Methyl ester*					
	12:0	*14:0*	*16:0*	*18:1*	*20:0*	*22:0*
			% of possible peak area			
Slight	95	100	100	100	100	100
Medium	53	76	88	88	100	100
High	46	61	73	84	89	100
Very high	6	37	64	81	86	100

The errors due to overloading increase with sample size and are such as to completely invalidate the analysis. They are greatest in the more volatile components.

The measurements of R_F values

With the equipment described above, nearly symmetrical peaks are obtained with the exception of the negative air peaks, which are asymmetric. However, under other conditions asymmetric peaks may be obtained (*cf.* HAWKE, DUNKLEY AND HOOKER[3]; BEERTHUIS *et al.*[4]) and tailing may also take place. Under such conditions neither the use of peak apices nor of the midpoints of the base give an accurate R_F value. The method adopted in this laboratory is to extrapolate the straight portion of the sides of the peak to intersect the baseline and to bisect the base of the triangle obtained. The same procedure is carried out with the air peak. The distances between the midpoints thus obtained are used in the calculation of R_F values. This system gave excellent reproducibility of R_F values as determined with 5% APL on "Celite 545" columns (Table III) and when R_F values obtained with 20% polydiethylene glycol adipate (DEGA) on "Celite 545" are determined, they closely resemble the values of FARQUHAR *et al.*[2] extrapolated to 207°. This is shown in Table II. The R_F values were independent of the shape of the peaks.

Table III comprises R_F values (relative to methyl stearate and adjusted to the air

TABLE II

R_F VALUES OFFATTY ACID ESTERS (RELATIVE TO METHYL STEARATE) ON DEGA COLUMNS AT 207° AND VALUES OBTAINED BY FARQUHAR *et al.*[2] EXTRAPOLATED TO 207°

Determination	*Methyl ester*			
	14:0	*16:0*	*18:1*	*20:0*
1	0.324	0.565	1.12	1.76
2	0.321	0.568	1.13	1.72
3	0.335	0.579	1.14	1.75
4	0.333	0.579	1.13	1.78
5	0.322	0.570	1.12	1.75
6	0.326	0.575	1.12	1.77
Mean	0.327	0.573	1.13	1.76
FARQUHAR *et al.*[2] at 207°	0.321	0.570	1.12	1.75

TABLE III

MEAN R_F VALUES OF METHYL ESTERS OF FATTY ACIDS DETERMINED ON A 5% APL ON "CELITE 545" COLUMN AT 207° AND STANDARD DEVIATIONS

Methyl ester	*R_F (air)/ 207° 18:0*	*S.D.*	*Methyl ester*	*R_F (air)/ 207° 18:0*	*S.D.*
11:0	0.061	0.004	18:3 (linoleic)	0.839	0.004
12:0	0.088	0.003	18:2 (linolenic)		—
13:0	0.140	0.003	18:1 (oleic)	0.879	0.004
14:0 iso	0.164	0.003	18:1 (elaidic)	0.901	0.001
14:0	0.203	0.007	18:0 iso	0.860	0.008
15:0 iso	0.263	0.005	18:0	1.000	—
15:0 anteiso	0.277	0.003	19:0	1.48	0.02
15:0	0.308	0.010	20:4 (arachidonic)	1.51	0.01
16:0 iso	0.390	0.006	20:0	2.17	0.05
16:1	0.394	0.005	21:0	3.20	0.05
16:0	0.456	0.007	22:1 (erucic)	4.28	0.07
17:0 iso	0.579	0.005	22:0	4.58	0.07
17:0 anteiso	0.603	0.003	23:0	6.87	0.18
17:0	0.681	0.011	24:0	10.2	0.3
			26:0	21.5	0.7

peak) of fatty acid methyl esters obtained with a 5 % APL on "Celite 545" column at 207°. The values quoted represent the means of 6 to 20 determinations. The esters are abbreviated as by FARQUHAR *et al.*[2].

It is well known that there is a linear relationship between log R_F and the number of carbon atoms in a homologous series of fatty acids. It is thus possible to derive simple empirical equations for the calculation of log R_F of any ester. Thus for:

n-saturated acid esters : $\log R_{F}n = 0.169n - 3.05$
(+)-anteiso acid esters : $\log R_{F}n = 0.169n - 3.10$
iso acid esters : $\log R_{F}n = 0.169n - 3.12$ and
cis monoenoic acid esters : $\log R_{F}n = 0.129n - 2.99$

where n represents the number of carbon atoms in the acid.

Similarly, linear relationships have been observed between R_F values and temperature (*cf.* FARQUHAR *et al.*[2]). Some changes of R_F value as determined with 5 % APL columns are shown in Table IV.

TABLE IV

CHANGES OF R_F WITH TEMPERATURE USING 5 % APL ON "CELITE 545" COLUMNS

Methyl ester	*R_F (air)/18:0 at*			
	*150°**	*180°**	*207°*	*225°**
12:0	0.056	0.076	0.088	—
14:0	0.146	0.170	0.203	0.222
16:0	0.379	0.398	0.456	0.474
18:0	1.000	1.000	1.000	1.000
20:0	—	2.22	2.17	2.06

* Determined on PYE Argon Chromatogram.

Acknowledgements

I wish to express my indebtedness to Messrs. D. R. BODY, S. ADHIKARI and B. A. NORTH of this laboratory for their assistance.

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Received March 30th, 1961

Gas chromatography of methyl derivatives of some barbiturates

With the increasing therapeutic use of barbiturate mixtures, it would be valuable to have an analytical method in forensic chemistry for identifying individual acids in these mixtures. Paper chromatography has been used for the purpose[1] but hitherto it has not been possible to separate the various malonylureas by gas chromatography because they decompose at high temperatures. JANÁK[2] made use of this property by separating the pyrolytic products of the acids by gas chromatography. Characteristic elution patterns were obtained with single barbiturates but the method does not lend itself to identification of individual acids in a mixture since several of the breakdown products were common to several acids.

Experiments carried out with 1,3-dimethyl derivatives of the barbituric acids showed that these compounds were thermostable and in this paper a method is described for separating these derivatives by gas chromatography.

Experimental

Initial attempts to separate the barbituric acids by gas chromatography proved unsuccessful, but after methylation with diazomethane satisfactory elution patterns were obtained. The 1,3-dimethyl derivatives were prepared as follows:

An excess of diazomethane in ethereal solution was poured on to the solid barbituric acid or its sodium salt in a test tube and allowed to stand overnight at room temperature in the fume cupboard. The ethereal solution was washed twice with an equal volume of saturated aqueous sodium bicarbonate, dried over anhydrous sodium sulphate and filtered. The ether was evaporated under reduced pressure leaving the methylated barbiturates either as viscous liquids or solids of low melting point.

Gas chromatography

Both apiezon L and Polyethylene Glycol Adipate[3] were used as stationary phases. The column support was Celite 545 prepared as described by ORR AND CALLEN[4]. The proportions, mesh sizes of the column pack and the operating conditions are as described in Table I. Columns of 130 cm by 4 mm were packed by vibration. The carrier gas was Argon and a Strontium 90 ionisation detector[5] was used. Samples were transferred to the columns with a micro pipette, the solid specimens being melted on the boiling water bath.

TABLE I

Stationary phase	*Mesh size of Celite 545*	*% stationary phase to support medium*	*Column working temperature °C*	*Argon*	
				Inlet pressure cm Hg	*Flow rate ml/min*
Apiezon L	60/80	15	197	96	86
Polyethylene glycol adipate	30/60	10	180	40	46

Retention volumes were calculated relative to Quinalbarbitone and the results are shown in Table II. Typical elution patterns are shown in Figs. 1 and 2.

TABLE II

Barbiturate		*Relative retention volumes*	
Pharmaceutical name	*Chemical name*	*Apiezon*	*Polyethylene glycol adipate*
Barbitone	5,5-Diethylbarbituric acid	0.37	0.51
Allobarbitone	5,5-Diallylbarbituric acid	0.48	0.72
Butobarbitone	5-Ethyl-5-*n*-butyl-barbituric acid	0.63	0.72
Amylobarbitone	5-Ethyl-5-isoamyl-barbituric acid	0.72	0.72
Pentobarbitone	5-Ethyl-5-(1-methylbutyl)-barbituric acid	0.87	0.85
Quinalbarbitone	5-Allyl-5-(1-methylbutyl)-2-barbituric acid	1.0	1.0
Hexobarbitone	5-(1-Cyclohexen-1-yl)-3,5-dimethyl-barbituric acid	1.98	3.87
Cyclobarbitone	5-Ethyl-5-(1-cyclohexen-1-yl)-barbituric acid	2.3	3.31
Phenyl-methyl-barbituric acid	5-Methyl-5-phenyl-barbituric acid	1.98	6.35
Phenobarbitone	5-Ethyl-5-phenyl-barbituric acid	2.3	5.38
Thiopentone	5-Ethyl-5-(1-methylbutyl)-2-thio-barbituric acid	3.76	1.41
		0.87	0.85
		1.98	3.87
		2.3	5.38
			3.31

Discussion

In order to identify the full range of barbiturates selected, two types of stationary phase were necessary for gas chromatography. The first, Apiezon L, separated completely those barbiturates with different alkyl substituents in the 5,5 positions according to chain length, those with shorter side chains being eluted earlier. Branching of the chain had the effect of shortening it so that Amylobarbitone was eluted before Pentobarbitone. On polyethylene glycol adipate the resolution of these alkyl derivatives was poor and Allobarbitone, Amylobarbitone and Butobarbitone emerged as a single peak.

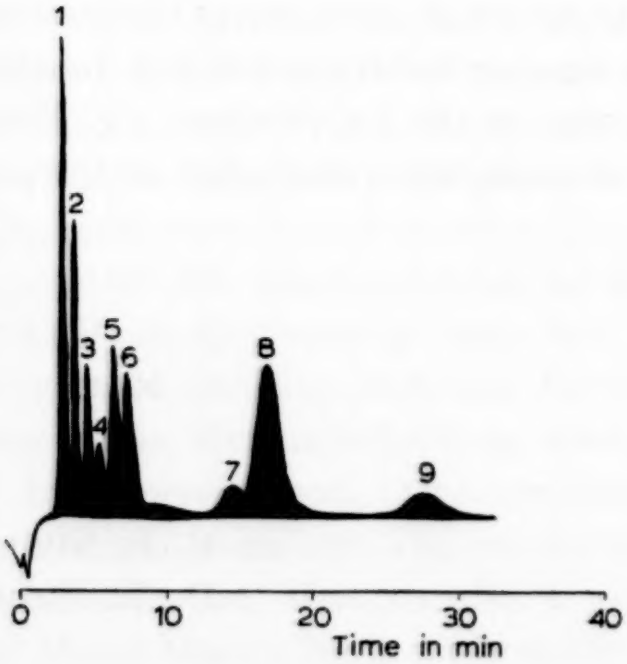

Fig. 1. Elution pattern on Apiezon L. 1 = Barbitone; 2 = Allobarbitone; 3 = Butobarbitone; 4 = Amylobarbitone; 5 = Pentobarbitone; 6 = Quinalbarbitone; 7 = Hexabarbitone; 8 = Phenobarbitone; 9 = Thiopentone.

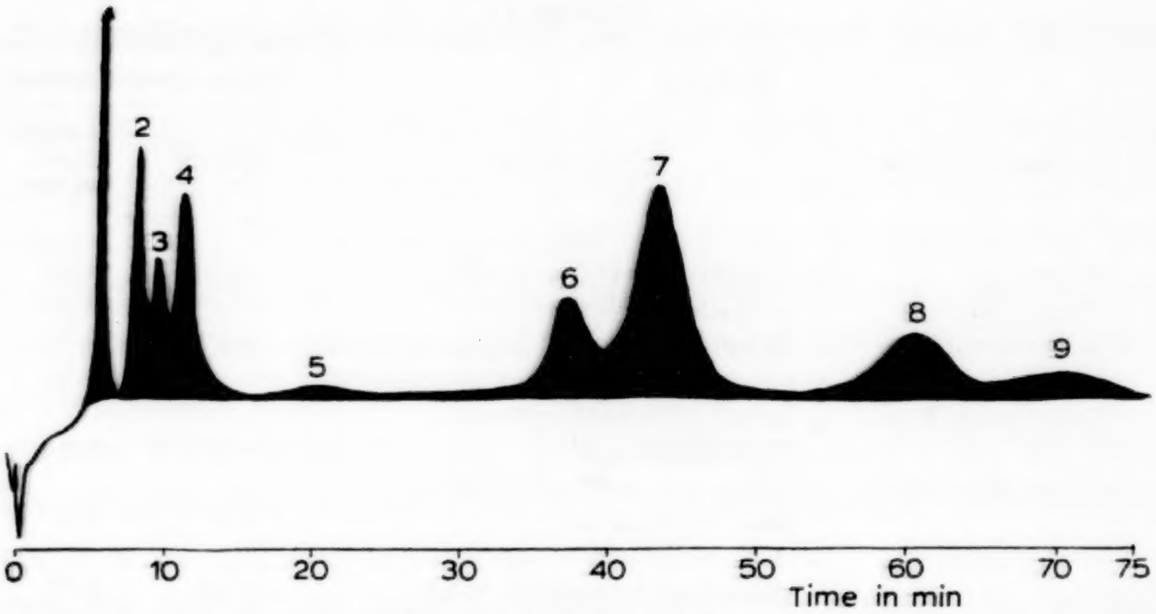

Fig. 2. Elution pattern on polyethylene glycol adipate. 1 = Barbitone; 2 = Allobarbitone, Butobarbitone, Amylobarbitone; 3 = Pentobarbitone; 4 = Quinalbarbitone; 5 = Unidentified impurity; 6 = Cyclobarbitone; 7 = Hexobarbitone; 8 = Phenobarbitone; 9 = Phenyl-methyl-barbituric acid.

On both types of column Thiopentone gave more than one peak, only one of which was characteristic. On Apiezon L it gave four peaks three of which were coincident with those of Pentobarbitone, Hexobarbitone and Phenobarbitone. On glycol adipate there was also a fifth peak in the Cyclobarbitone position. The occurrence of a peak in the Pentobarbitone position is readily explained since it is the oxygen analogue of Thiopentone but the reason for the occurrence of the other peaks is not clear, possibly they are due to pyrolytic decomposition of the more labile sulphur analogue.

Separation of phenyl and cyclohexenyl derivatives was only achieved by using polyethylene glycol adipate. Separation on this type of column depends on polarity with the result that the cyclical barbiturates emerged in the following order: Cyclobarbitone, Hexabarbitone, Phenobarbitone and Rutanol. On Apiezon L Cyclobarbitone and Phenobarbitone emerged as a single peak after the combined Hexobarbitone and phenyl-methyl-barbituric acid peak.

Methylation takes place by substitution in the 1 and 3 positions of the barbituric acid nucleus. For this reason it is impossible to separate barbituric acid and N-methylbarbituric acid derivatives with identical groups in the 5,5 positions, *e.g.* Phenobarbitone and Prominal. Ethylation instead of methylation may allow such separations but this has not yet been explored.

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Received April 28th, 1961

Notes

Paper chromatography of 2,4-dinitrophenylhydrazones. Extension of the Huelin method

During the course of certain studies on the carbonyl components of cigar tobacco smoke, it became necessary to separate these compounds as their 2,4-dinitrophenylhydrazones (DNPH's). For this purpose the paper chromatographic method of HUELIN[1] was selected and used with only slight modifications.

Admittedly, there have been newer methods for 2,4-dinitrophenylhydrazones published since the work of HUELIN and, as MACEK states[2], this method may no longer be in great use. Initially, several of these newer techniques were tried, but they failed to give satisfactory results in our hands. For example, one method[3] failed to give sufficient movement or discrete spots, while another[4] was not reproducible due to lack of information from the authors. The precoated-paper systems[5–7] seemed too troublesome to control. In contrast, the method of HUELIN was found to be very simple, highly reproducible and provided good separation and spot formation.

To establish R_F values under our own conditions, some 25 known DNPH's were investigated. Since HUELIN's paper dealt with only a limited number of DNPH's, it seemed advisable to submit our data as an extension of the original work. These are presented in Table I. Each R_F value listed is the average of several runs on different days. The reproducibility was generally good, varying only a few hundredths, except in the cases where a range is given. The only compound presenting any difficulty was propanal-DNPH, which exhibited extremely poor reproducibility.

The variation between these data and those of HUELIN may be attributed to differences in operating conditions. The temperature was probably the most significant factor in this; HUELIN failed to mention any operating temperature.

In the present work, chromatograms were run in a cylindrical glass jar 22 cm in diameter and 46 cm high. The solvent container at the bottom was a crystallizing dish 15 × 7.5 cm in size. Whatman No. 1 paper was used in sheets 42 × 30 cm. Samples were placed along a line 2 cm from the bottom and 3 cm apart. The solvent system

TABLE I

R_F VALUES OF AUTHENTIC 2,4-DINITROPHENYLHYDRAZONES

Ketone-DNPH	R_F	*Aldehyde-DNPH*	R_F
Acetone	0.41	Formaldehyde	0.17
Methyl ethyl	0.55–0.59	Acetaldehyde	0.27
Methyl isopropyl	0.69	Propanal	0.37–0.48
Methyl isobutyl	0.76	Butanal	0.49
Methyl *n*-amyl	0.83–0.87	Heptanal	0.84
Diisopropyl	0.85–0.91		
Di-*n*-propyl	0.94		
Diisobutyl	0.94		

Unsaturated-DNPH	R_F	*Dicarbonyl-DNPH*	R_F
Crotonal	0.41	Acetylacetone (mono)	0.40
2-Hexenal	0.65	Dilevulinic acid (mono)	0.45
2-Heptenal	0.77	Glyoxal (bis)	0.00
2-Nonenal	0.92	Methyl glyoxal (bis)	0.00
Phorone	0.85	Dimethyl glyoxal (bis)	0.00
		Acetonylacetone (bis)	0.00
		3-Hexene-2,5-dione (bis)	0.00

consisted of heptane–methanol (2:1, v/v). (HUELIN used a petroleum fraction boiling between 95° and 105° in place of heptane.). This mixture was shaken and allowed to separate into two phases. At 25°, the upper, heptane-rich phase was 57 %, while the lower, methanol-rich phase was 43 % of the total volume. The upper phase was used for development and the lower phase for equilibration, as prescribed by HUELIN. The remainder of the procedure followed the original work except that the temperature was maintained at 25 ± 1°.

Instead of the usual alkali spray for detection of spots on the completed chromatograms, observation under ultraviolet light was employed. This proved to be a more sensitive technique.

Acknowledgement. This work was supported in part by funds made available by the Cigar Manufacturers Association of America, Inc.

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Agricultural Research Service, United States
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Received January 24th, 1961

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Two improved methods for the separation of 2,4-dinitrophenylhydrazones of carbonyl compounds

During our work the need was felt for a rapid method for the determination of certain carbonyl compounds. We therefore applied the method evolved by HORNER AND KIRMSE[1], which was later modified by GASPARIČ AND VEČEŘA[2]. To accelerate this method the apparatus for centrifugal chromatography, described by PAVLÍČEK AND DEYL[3], was used. At the same time we separated these substances on chromatoplates using the modified technique described by MOTTIER[4].

Standards of the 2,4-dinitrophenylhydrazones of the carbonyl compounds were prepared from aqueous solutions of pure substances by precipitation with 0.5 % solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid.

1. *Separation on chromatoplates*

The chromatographic separation was carried out on a layer of aluminium oxide (degree of activity II–III), which was prepared by coating a glass plate with a layer of 0.8–0.9 mm thickness, according to the procedure described by MOTTIER[4]. Development by the ascending method was employed, the glass plate being inclined at an angle of 15–20° to the horizontal plane. Two different solvent systems were chosen as mobile phases, ether (only once distilled and not dried) and the mixture benzene–hexane (1:1). Using a layer of 25 cm length, the time for development is about 15 minutes. Samples in the form of a 2 % solution in chloroform were applied by the following procedure: 0.1 ml was spotted for analysis, or 0.5–0.75 ml as a 4 cm long line for preparative purposes.

The R_F values are given in Table I and the appearance of the chromatogram is shown in Fig. 1.

2. *Centrifugal separation*

Chromatography in a centrifugal field was carried out using Whatman No. 3 paper, impregnated with a 25 % solution of dimethylformamide in ethanol. The chromatographic paper was dried at room temperature by a stream of cold air. Samples of the 2,4-dinitrophenylhydrazones were spotted on the start, which was about 1.5 cm from the centre of rotation. Aliquots of the sample of 10–20 μl were applied on the start in the form of a 2% solution in chloroform.

The chromatograms were developed with cyclohexane, and the tank was saturated with both the mobile and the stationary phases; when a paper disc of 20 cm diameter was used at 20° and 750 r.p.m. the developing time was about 30 min. The overflowing technique was applied with great success for the separation of formaldehyde and acetaldehyde (*i.e.* until the indicating spot of acetone 2,4-dinitrophenylhydrazone reaches the front of the paper, see Fig. 2). This separation takes 50 minutes on an average.

Only those 2,4-dinitrophenylhydrazones were separated whose R_F values have

TABLE I

R_F VALUES OF SOME 2,4-DINITROPHENYLHYDRAZONES OF CARBONYL COMPOUNDS, USING THE CHROMATOPLATE TECHNIQUE

Carbonyl compound	*Solvent system*	
	Ether	*Benzene–hexane (1:1)*
Formaldehyde	0.71	0.36
Acetaldehyde	0.87	0.38
Pentanal	0.90	0.76
Furfural, *cis*	0.86	diffuse zone
Furfural, *trans*	0.76	
Benzaldehyde	diffuse zone	0.40
Acetone	0.86	0.34
Methyl ethyl ketone	0.98	0.45
Cyclohexanone	0.93	0.70
Glyoxal	0.49	0.70
Methylglyoxal	0.46	0.67
Lævulic acid	0.00	0.00

been determined by the ascending technique and partially the descending technique[1,2]. The R_F values obtained with centrifugal chromatography are given in Table II.

Rapid and accurate marking of the front is of great importance in the determination of R_F values. Fig. 3 shows also a typical example of the separation of 2,4-dinitrophenylhydrazones by centrifugally accelerated chromatography. It can be seen

TABLE II

R_F VALUES OF SOME 2,4-DINITROPHENYLHYDRAZONES OF CARBONYL COMPOUNDS, USING DESCENDING AND CENTRIFUGAL DEVELOPMENT

Carbonyl compound	*Descending development*		*Centrifugal development**
	HORNER AND KIRMSE[1]	GASPARIČ AND VEČEŘA[2]	
Formaldehyde	0.17	0.20	0.45
Acetaldehyde	0.27	0.32	0.63
Pentanal	—	—	0.92
Heptanal	0.83	0.82	0.94
Furfural, *cis*	—	0.26	0.61
Furfural, *trans*	—	0.12	0.40
Acetone	0.43	0.48	0.67
Methyl ethyl ketone	0.56	0.66	0.83
Cyclohexanone	0.70	0.77	0.93
Laevulic acid	—	—	0.05
Acetoacetic acid ethyl ester	—	—	0.50

* Whatman No. 3 paper.

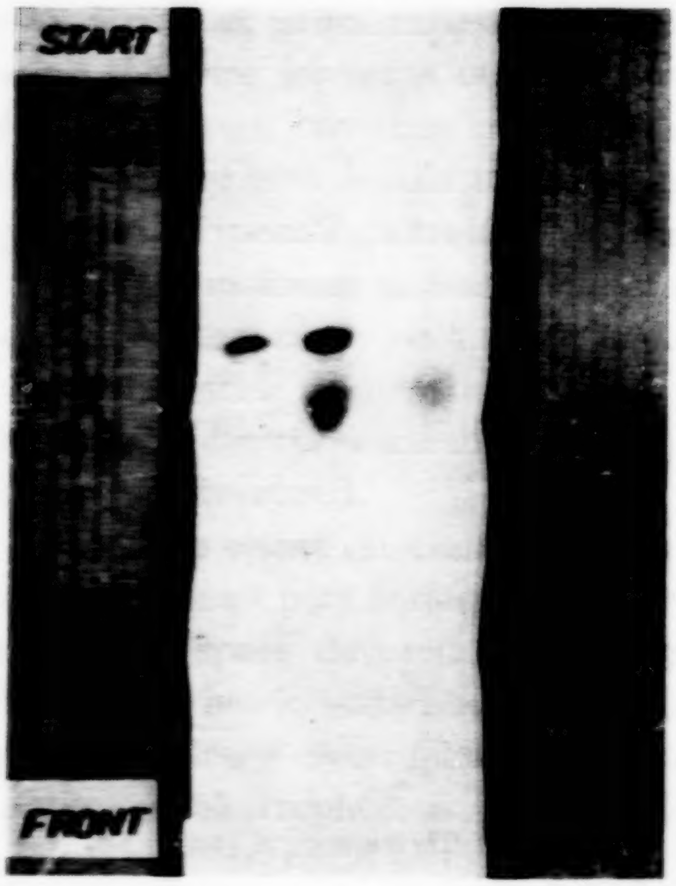
START
FRONT

Fig. 1. Chromatogram of a simple mixture of 2,4-dinitrophenylhydrazones. From left to right: formaldehyde, formaldehyde + methyl ethyl ketone (mixture); methyl ethyl ketone.

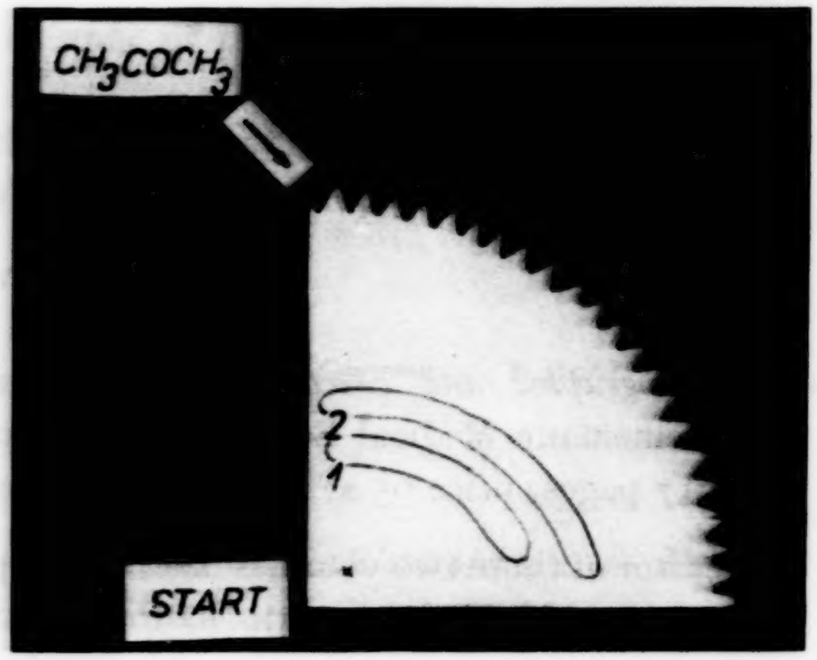
CH₃COCH₃
2
1
START

Fig. 2. Separation of a mixture of the 2,4-dinitrophenylhydrazones of formaldehyde (1) and acetaldehyde (2) (overflowing technique).

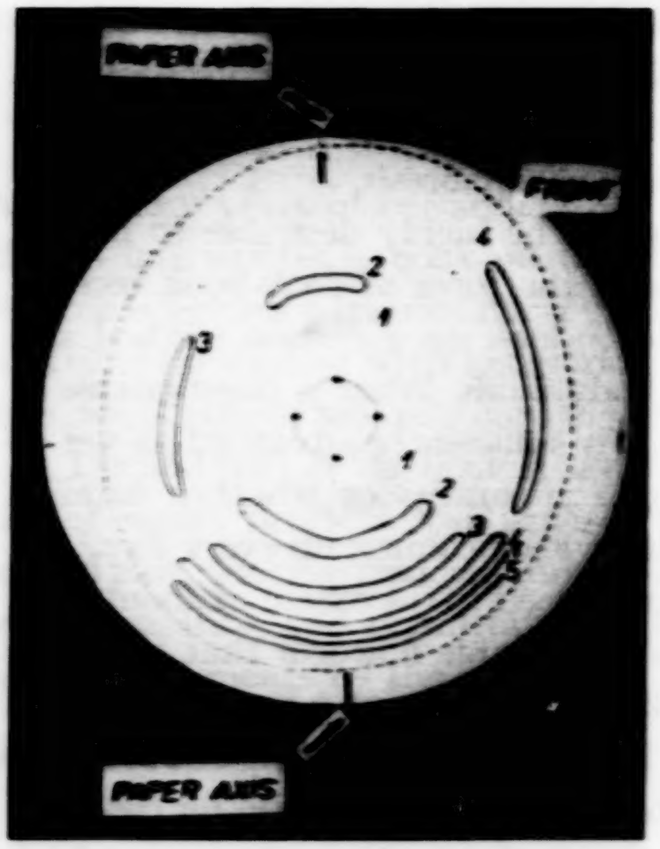
PAPER AXIS
FRONT
1
2
4
1
3
1
2
3
4
5
PAPER AXIS

Fig. 3. Separation of a mixture of the 2,4-dinitrophenylhydrazones of some carbonyl compounds, using centrifugal chromatography. (1,2) furfural, *cis* + *trans*; (3) acetone; (4) methyl ethyl ketone; (5) cyclohexanone.

from this figure that the quality of separation is much better than that obtained with modifications of centrifugal chromatography where the separation is based on adsorption.

Acknowledgement

The authors wish to express their deep gratitude to Dr. MACEK, Research Institute of Pharmacy and Biochemistry, Prague, for the interest he has shown in this work.

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Received April 6th, 1961

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J. Chromatog., 6 (1961) 187–190

BOOK REVIEWS

Chromatographic and Electrophoretic Techniques, edited by IVOR SMITH, William Heinemann Medical Books Ltd., London, 1960; Vol. 1: *Chromatography*, xv + 617 pages, price 65 s; Vol. 2: *Zone Electrophoresis*, viii + 215 pages, price 30 s.

The editor of these two volumes has tried to prepare a practical handbook for chromatographic and electrophoretic methods that contains sufficient details for work without consultation of the literature to be possible. He was aided in this task by 22 authors for chromatography and 8 authors for electrophoresis.

We agree entirely with the editor that it would be highly desirable to have a volume that presents chromatographic methods in the form of cook-book recipes, so that a technician or collaborator may be told to look up a certain method and apply it directly to, for example, amino acids in body fluids. What is questionable at the moment is whether work on chromatography is sufficiently advanced as yet to permit the suggestion, for example of one single apparatus or whether detailed methods can be given for fields where new problems keep coming up every day.

It may be said, however, that the two books succeed to the extent of about 90% in providing good and detailed techniques. It is regrettable however that there are several techniques and whole chapters that look rather perplexing to the reviewer. We shall pick out a few: on page 18 (Vol. 1) it is suggested that samples should be placed on the paper with a platinum loop, and the platinum wire flamed for cleaning. No limitations of this technique are mentioned and the reviewer hates to think what would happen if this technique were applied indiscriminately by a beginner in inorganic or radiochemistry, for example for placing ^{103}Pd onto a paper and then using the wire for some other radioactivity. It is of course common sense not to do such things, but then most techniques of paper chromatography are common sense, so why give details at all unless they are absolutely foolproof. Then there are some rather puzzling chapters, for example that on circular chromatography and that on ion-exchange papers. Circular chromatography is as usual described as space saving (is there really such a housing shortage?) but leaves the beginner in doubt as to whether after reading this chapter or that on ion-exchange papers he should not forget all about the usual sheet chromatography and from now on work only with the technique advertised.

In the volume on electrophoresis we welcome very much the fact that only a relatively small part is devoted to the separation of serum proteins on paper strips and much space devoted to haemoglobins, cellulose acetate electrophoresis and starch (gel, block, etc.) electrophoresis. There is also a short but adequate chapter on high voltage electrophoresis and another short but less adequate chapter on continuous electrophoresis. The mention of some of the commercial types of continuous apparatus would seem essential.

In spite of these few shortcomings these books can be strongly recommended to anyone intending to use chromatographic and electrophoretic techniques in clinical and biochemical problems.

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J. Chromatog., 6 (1961) 190–191

Estudio de la Proteinas del Suero Humano por Medio de la Electroforesis en Papel, by JUAN A. MORALES MALVA, Editorial Universitaria, Santiago de Chile, 1958, xix + 169 pages.

After many years of research in the field of haematology Prof. JUAN A. MORALES MALVA has written a book on the study of proteins by electrophoresis. This small volume is neither a treatise nor a guide to electrophoresis, but a personal and critical contribution dealing with his work on the use of this technique in the diagnosis of diseases. Methods are only considered as a means to diagnostic work.

The first part of the book (pages 1–52) deals with an examination of the properties of serum proteins, particularly those in abnormal and pathological sera. The second part, which is divided into two sections, describes the techniques of electrophoretic separation and quantitative determination, and a series of previously unpublished experiments by the author.

G. B. MARINI-BETTÒLO (Rome)

J. Chromatog., 6 (1961) 191

Aluminiumoxyd "Fluka" für Säulen- und Dünnschicht-Chromatographie und für Trocknung von Lösungsmitteln (*Fluka-Bulletin*, 1 (1961)), Fluka AG, Buchs, S.G., Switzerland.

This 10 page bulletin deals with the fields of application of a series of alumina preparations made especially for chromatography. The firm of Fluka has set itself the aim of producing alumina of constant quality and suited for specific purposes, such as column chromatography (basic, neutral or acid), chromatoplates (special particle size) and for drying (a moisture indicator incorporated in the alumina). The bulletin surveys the literature of these applications quoting short abstracts from the original papers or giving a suitable bibliography. We have the impression that the alumina as well as the bulletin will be welcome in many laboratories.

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Announcement

PRELIMINARY ANNOUNCEMENT OF 1962 PITTSBURGH CONFERENCE

The Thirteenth Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy will be held at the Penn-Sheraton Hotel in Pittsburgh, Pa., U.S.A., March 5–9, 1962. Approximately 200 papers on all phases of analytical chemistry and spectroscopy will be presented. A symposium entitled RAMAN SPECTROSCOPY will be sponsored jointly with the Coblentz Society, and a symposium entitled VACUUM SPECTROSCOPY will be co-sponsored with the Society for Applied Spectroscopy. Symposia entitled GAS CHROMATOGRAPHY, SPECTROSCOPIC STUDIES OF POLYMERS, and CHEMICAL ANALYSIS OF METALS will also be held.

Original papers on all phases of analytical chemistry and spectroscopy are invited. A brief abstract (150 words) of each paper will be printed in the program. Three copies of this abstract, with a letter listing the names of the authors, the laboratory in which the work was done, and the current addresses of the authors, should be addressed to:

Dr. CHARLES E. GLICK, Program Chairman,
The Thirteenth Pittsburgh Conference,
Applied Research Laboratory,
United States Steel Corporation,
Monroeville, Pa., U.S.A.

Final date for receipt of abstracts is October 16, 1961. One copy of the complete paper must be submitted by January 11, 1962.

In addition to the program of technical papers, there will be an exhibition of the newest analytical instrumentation. More than 110 companies will display instruments, chemicals, and equipment.

J. Chromatog., 6 (1961) 192

A LUMPED-FILM MODEL FOR GAS-LIQUID PARTITION CHROMATOGRAPHY

PART I. NUMERICAL METHODS OF SOLUTION

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(Received December 21st, 1960)

Two theoretical approaches have been taken to gas-liquid partition chromatography (G.L.C.)—the first may be termed the plate theory and the second the rate theory. The major results of both theories have been summarized by KEULEMANS[1]. The plate theory or H.E.T.P. method has been used successfully to analyze experimental data by MARTIN AND SYNGE[2], MAYER AND TOMPKINS[3], GLUECKAUF[4] and VAN DEEMTER, ZUIDERWEG AND KLINKENBERG[5]. The main drawback of the plate theory is that the H.E.T.P. is a semi-empirical quantity that is calculated from the elution curve and although it serves as a concept by which the experimental data may be analyzed and explained, it is not a quantity fundamental enough to be derived solely from the physical properties of the system. On the other hand, the rate theory begins by formulating the partial differential equations governing G.L.C. such that the physical properties of the system occur as coefficients. Because the resulting equations are too complex to solve analytically it is usual to make three simplifying assumptions.:

1. The equilibrium concentrations in the gas and liquid phases are directly proportional (linear chromatography).
2. Equilibrium between liquid and vapor phases is established immediately (instantaneous equilibrium).
3. Axial diffusion may be neglected.

Chromatography subject to the above assumptions is commonly called ideal chromatography, and theories of ideal chromatography have been developed by BOYD, ADAMSON AND MYERS[6], LAPIDUS AND AMUNDSON[7], KLINKENBERG[8], and THOMAS[9]. GIDDINGS AND EYRING[10], however, have treated the diffusional processes involved by the random walk method, thus obtaining an interesting molecular theory of chromatography. The main drawback to the rate theory, apart from the above assumptions, is that, in order to simplify the differential equations for solution, it is necessary to introduce an arbitrary rate constant, usually a mass transfer coefficient or a reaction velocity constant, whose value is obtained by fitting the analytical

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J. Chromatog., 6 (1961) 193–208

solution to the elution curve. However, VAN DEEMTER *et al.*[5] have discussed a method of estimating the rate constant from the physical properties of the system, subject to the assumptions of ideal chromatography.

The object of the present work is to remove some or all of the restrictions involved in the assumption of ideal chromatography and to devise a method, based on the rate theory, to predict the shape and position of the elution curve from the physical properties of the system alone without the use of an arbitrary rate constant. This has been accomplished (*cf.* FUNK AND HOUGHTON[11]) by using a lumped-film model for the liquid phase. All three restrictions involved in the assumption of ideal chromatography are removed by programming the differential equations on an IBM-704 digital computer using finite difference methods. The computer solution can also be used to account for the effects of nonlinear solubility and column pressure drop. Analytical solutions will also be reported in Part II for the cases where the restrictions of instantaneous equilibrium and/or no longitudinal dispersion are removed.

THE PARTIAL DIFFERENTIAL EQUATIONS OF NON-IDEAL CHROMATOGRAPHY

There are three phases present in G.L.C.—a flowing vapor phase and a stationary involatile liquid phase coated on to an impermeable solid phase. A binary system is assumed with a solute and carrier gas in the vapor phase and solute and solvent in the liquid phase. The one-dimensional continuity equation for the vapor phase is:

$$\frac{\partial X}{\partial t} = E\frac{\partial^2 X}{\partial z^2} - \left(u - \frac{2E}{P}\frac{\partial P}{\partial z}\right)\frac{\partial X}{\partial z} - \frac{k_G RTA}{\varepsilon}(X - X_i) + \frac{EX}{P}\frac{\partial^2 P}{\partial z^2} \qquad (1)$$

If the gas flow in the packed bed is laminar and unidirectional, then Darcy's law may be applied as follows:

$$u = \frac{KP}{\eta}\frac{\mathrm{d}P}{\mathrm{d}z} \qquad (2)$$

Also for compressible flow:

$$uP = u_i P_i \qquad (3)$$

Solution of eqns. (2) and (3) yields (*cf.* KEULEMANS[1]):

$$P = \sqrt{P_i^2 - \frac{z}{L}(P_i^2 - P_o^2)} \qquad (4)$$

whence:

$$\frac{\partial P}{\partial z} = -\frac{1}{P}\frac{(P_i^2 - P_o^2)}{2L} \qquad (5)$$

$$\frac{\partial^2 P}{\partial z^2} = \frac{1}{P^3}\left(\frac{P_i^2 - P_o^2}{2L}\right)^2 \qquad (6)$$

For the liquid phase it is assumed that the diffusion coefficient, D_L, is independent of concentration and that diffusion in the thin uniform film of partitioning liquid is unidirectional, so that:

$$\frac{\partial c}{\partial t} = D_L \frac{\partial^2 c}{\partial y^2} \tag{7}$$

If it is assumed that there is a gas film resistance of the Whitman type at the interface between the liquid and vapor phases, the concentration changes are depicted in Fig. 1 as a function of time as the solute passes into the liquid phase and is eluted out again. The boundary conditions to be applied to eqn. (7) may be obtained by referring to Fig. 1. and are as follow:

$$\left(\frac{\partial c}{\partial y}\right)_{y=h} = 0 \tag{8}$$

$$D_L \left(\frac{\partial c}{\partial y}\right)_{y=0} = k_G P (X - X_i) \tag{9}$$

where k_G is the Whitman gas film coefficient.

The final equation that couples the vapor phase and liquid phase equations is the equilibrium solubility isotherm:

$$c_i = K_1 P X_i + K_2 P^2 X_i^2 \tag{10}$$

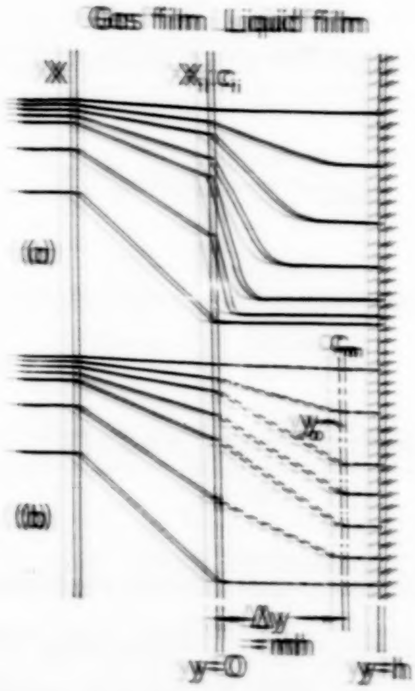

Fig. 1. Concentration distributions in the liquid film as a function of time; (a) variable depth of penetration, (b) assuming constant depth of penetration.

Equation (10) has a nonlinear term that allows for non-ideality in the liquid phase.

The general assumptions used in the derivation of the above equations are enumerated below:

(1) Uniform one-dimensional flow through a uniformly-packed bed.

(2) Longitudinal dispersion in the gas phase can be adequately represented by an eddy diffusivity, E, which is independent of position and concentration.

(3) The presence of a Whitman-type[12] gas film resistance, k_G, at the gas–liquid interface.

(4) The diffusion coefficient in the liquid phase, D_L, is independent of concentration.

(5) Pressure drops through the bed are governed by Darcy's law.

(6) Ideal gases and gas mixtures.

In order to conveniently solve the above equations, it is necessary to formulate a model that will eliminate the independent variable, y. This has been accomplished by representing the liquid film as a lumped system using finite difference techniques.

THE LUMPED-FILM MODEL FOR THE LIQUID PHASE

The function $c(z,y,t)$ can be expanded about some point y_0 in the liquid film by means of Taylor's series so that the second derivative becomes:

$$\frac{\partial^2 c(z,y,t)}{\partial y^2} = \frac{1}{\Delta y^2}\left[c(z,y_0 - \Delta y,t) - c(z,y_0,t)\right] -$$

$$- \frac{1}{\Delta y^2}\left[c(z,y_0,t) - c(z,y_0 + \Delta y,t)\right] \tag{11}$$

If Δy is always required to be the distance from the surface of the liquid film to the lumping point, y_0, then eqn. (9) may be approximated by:

$$D_L\left[\frac{c_i - c(z,y_0,t)}{\Delta y}\right] = k_G P(X - X_i) \tag{12}$$

If Δy is also the distance from the lumping point to the surface of the solid, eqn. (8) leads to the approximation:

$$\frac{c(z,y_0,t) - c(z,y_0 + \Delta y,t)}{\Delta y} = 0 \tag{13}$$

The only way for Δy to satisfy both of the above requirements is for m in Fig. 1 to be 1/2. In this case, eqns. (7), (11), (12) and (13) may be combined to give a single-section lumped-film model represented by the following differential-difference equation:

$$\frac{\partial c_m}{\partial t} = \frac{D_L}{\Delta y^2}(c_i - c_m) \tag{14}$$

where:

$$c_m = c(z,h/2,t)$$

and:

$$\Delta y^2 = h/2 \tag{15}$$

It is evident that a single-section approximation will only apply when the liquid film is thin, so that the depth of penetration is of the order of one-half the film thickness.

This argument leads to the possibility of m having an average value other than 1/2, and this case will be treated in Part III as the penetration model.

ELIMINATION OF INTERFACIAL CONCENTRATIONS FROM THE EQUATIONS

For the case of a nonlinear solubility isotherm, substitution of eqn. (12) into eqn. (10) eliminates c_i and leads to the following quadratic in X_i:

$$K_{13} X_i^2 + K_{14} X_i - (k_G P X + D_L c_m/\Delta y) = 0 \tag{16}$$

where:

$$K_{13} = \frac{D_L K_2 P^2}{\Delta y} \tag{17}$$

$$K_{14} = \frac{D_L K_1 P}{\Delta y} + k_G P \tag{18}$$

Solving for X_i we obtain:

$$X_i = -K_{15}/2 \pm \sqrt{K_{15}^2/4 + (k_G P X + D_L c_m/\Delta y)/K_{13}} \tag{19}$$

where:

$$K_{15} = K_{14}/K_{13} \tag{20}$$

Equation (19) may now be used to elimate X_i from eqn. (11) for the case of nonlinear solubility.

The case of a linear solubility isotherm may be obtained by letting $K_3 \rightarrow 0$ in eqn. (19) and using the positive sign in front of the square root, so that:

$$X_i = K_{16} X + K_{17} c_m \tag{21}$$

where:

$$K_{16} = k_G P/K_{14} \tag{22}$$

$$K_{17} = D_L/\Delta y K_{14} \tag{23}$$

Equation (21) may be used to eliminate X_i from eqn. (11) for the case of linear solubility.

INITIAL AND BOUNDARY CONDITIONS

Fig. 2 shows the conditions for an initial value problem that is closely allied to the experimental conditions actually found in G.L.C. The slug of solute of mole fraction X_0 is trapped between two gates that can be opened simultaneously at $t = 0^+$ to introduce the solute into the carrier gas stream. It is assumed that the flow is established instantaneously and that there are no end effects in the column. The initial

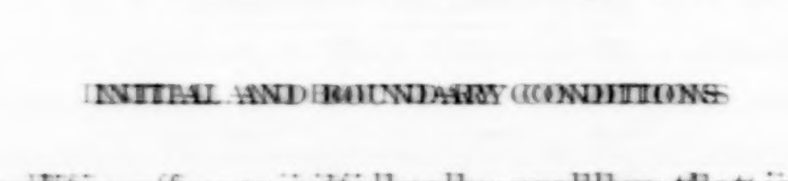

Fig. 2. The initial value problem of G.L.C.

conditions for this case are then:

$$X(z,0) = \begin{cases} X_0, & 0 < z < L_1 \\ 0, & z > L_1 \end{cases} \qquad (24)$$

$$c_i(z,0) = c_m(z,0) = 0, \; L_2 < z < L_3$$

and the boundary conditions are:

$$\text{Gas velocity} = \begin{cases} u, & L_2 < z < L_3 \\ u\varepsilon, & z < L_2 \text{ and } z > L_3 \end{cases} \qquad (25)$$

$$\text{Gas film coefficient} = \begin{cases} k_G, & L_2 < z < L_3 \\ 0, & z < L_2 \text{ and } z > L_3 \end{cases}$$

The solution to the problem is the mole fraction of solute in the gas phase passing the detector as a function of time, $X(L_4,t)$, and can be obtained by solving the differential equations in finite difference form. The numerical solution obtained completely removes the restrictions of ideal chromatography and can therefore be used to account for the effects of nonlinear solubility, finite diffusion rates, axial diffusion and column pressure drop.

FINITE DIFFERENCE EQUATIONS

In order to obtain the difference equations in a form explicit in the variables X and c_m, the finite difference approximations are made forward in time and backward in space as follows:

$$\frac{\partial^2 X}{\partial z^2} = \frac{X(i+1,j) - 2X(i,j) + X(i-1,j)}{\Delta z^2} \qquad (26)$$

$$\frac{\partial X}{\partial z} = \frac{X(i,j) - X(i-1,j)}{\Delta z} \qquad (27)$$

$$\frac{\partial X}{\partial t} = \frac{X(i,j+1) - X(i,j)}{\Delta t} \qquad (28)$$

$$\frac{\partial c_m}{\partial t} = \frac{c_m(i,j+1) - c_m(i,j)}{\Delta t} \qquad (29)$$

The finite difference equations are obtained by substituting eqns. (26), (27), (28) and (29) into eqns. (1) and (14) and using eqns. (19) or (21) to eliminate X_i. The results for nonlinear and linear isotherms are summarized below:

1. *Nonlinear solubility isotherm*

$$X(i,j+1) = \beta_1(P)X(i,j) + \beta_2(P)X(i-1,j) + \beta_3(P)X(i+1,j) +$$

$$+ \frac{k_G(i)RTA\Delta t}{\varepsilon}\left[-K_5(i)/2 + \sqrt{K_5^2(i)/4 + (k_G(i)P(i)X(i,j) + D_L c_m(i,j)/\Delta y)/K_3(i)}\right] \qquad (30)$$

$$c_m(i,j+1) = c_m(i,j) + \left[\frac{k_G(i)P(i)\Delta t}{\Delta y}\right] X(i,j) - \tag{31}$$

$$- \frac{k_G(i)P(i)\Delta t}{\Delta y}\left[- K_5(i)/2 + \sqrt{K_5^2(i)/4 + (k_G(i)P(i)X(i,j) + D_L c_m(i,j)/\Delta y)/K_3(i)}\right]$$

where:

$$\beta_1(P) = 1 - \frac{2E\Delta t}{\Delta z^2} - \frac{u(i)\Delta t}{\Delta z} + \frac{2E\Delta t}{\Delta z P(i)}\frac{\mathrm{d}P(i)}{\mathrm{d}z} - \frac{k_G(i)RTA\Delta t}{\varepsilon} + \frac{E}{P(i)}\frac{\mathrm{d}^2P(i)}{\mathrm{d}z^2} \tag{32}$$

$$\beta_2(P) = \frac{u(i)\Delta t}{\Delta z} - \frac{2E\Delta t}{\Delta z P(i)}\frac{\mathrm{d}P(i)}{\mathrm{d}z} + \frac{E\Delta t}{\Delta z^2} \tag{33}$$

$$\beta_3(P) = \frac{E\Delta t}{\Delta z^2} \tag{34}$$

2. *Linear solubility isotherm*

$$X(i,j+1) = \beta_2(P)X(i-1,j) + \beta_3(P)X(i+1,j) + \beta_4(P)X(i,j) + \beta_5(P)c_m(i,j) \tag{35}$$

$$c_m(i,j+1) = \beta_6(P)c_m(i,j) + \beta_7(P)X(i,j) \tag{36}$$

where:

$$\beta_4(P) = 1 - \frac{2E\Delta t}{\Delta z^2} - \frac{u(i)\Delta t}{\Delta z} + \frac{2E\Delta t}{\Delta z P(i)}\frac{\mathrm{d}P(i)}{\mathrm{d}z} + \frac{E}{P(i)}\frac{\mathrm{d}^2P}{\mathrm{d}z^2} -$$

$$- K_6(i)AD_LK_1RT\Delta t/\Delta y\varepsilon \tag{37}$$

$$\beta_5(P) = K_7(i)k_G(i)ART\Delta t/\varepsilon \tag{38}$$

$$\beta_6(P) = 1 - K_6(i)D_L\Delta t/\Delta y^2 \tag{39}$$

$$\beta_7(P) = K_6(i)P(i)D_LK_1\Delta t/\Delta y^2 \tag{40}$$

The coefficients $\beta(P)$ are functions of pressure and therefore eqns. (3), (4), (5) and (6) must be utilized to evaluate the pressure dependence.

The above equations may be solved by dividing the chromatographic column into axial increments, Δz, such that $z = i\Delta z$, and using time increments Δt such that $t = j\Delta t$. The numerical solution is then obtained by a marching process, starting with the initial conditions and using two time–distance grids, one for X and the other for c_m. The equations have been programmed in Fortran language for an IBM-704 digital computer.

STABILITY AND CONVERGENCE OF THE NUMERICAL SOLUTION

In the numerical solution of partial differential equations by finite differences, the concepts of stability and convergence are very important. The term convergence is used to denote how close the solution of the difference equations approaches the exact solution of the differential equations in the limit as the size of the increments (Δz and Δt) is reduced to zero. The relationship between Δz and Δt is determined, however, by the stability criterion. If the error is defined as the departure of the numerical solution from the exact solution, then stability deals with the growth and amplification of this error.

The cause of instability in the numerical solution of partial differential equations has been discussed by a number of writers[13–15], some of whom attribute instability to round-off errors, while others claim that it is inherent in the difference equations. RICHTMYER[15] has shown that in the numerical solution of the simple parabolic heat equation, instability may not be concerned with round-off errors, but rather is the property of the particular system of difference equations. The same view is taken in the present work, even though the same formal results can be achieved by considering the problem as one of propagation of round-off error, as discussed by HOUGHTON[16].

A differential equation is said to be unstable if it yields an unbounded output for a bounded input. This behavior is an inherent characteristic of the equation. To extend this concept to difference equations, it is only necessary to state that a stable solution will result if the coefficients of the difference equations are fixed so that the output is bounded for any bounded input. The fact that the bounded input may be round-off error (among other forms of disturbance) provides a connection with the round-off method of determining instability. For the case of constant pressure ($dP/dz = 0$ and $d^2P/dz^2 = 0$) with a linear solubility isotherm, eqns. (35) and (36) become:

$$X(i,j+1) = \alpha_2 X(i-1,j) + \alpha_3 X(i+1,j) + \alpha_4 X(i,j) - \alpha_5 c_m(i,j) \tag{41}$$

$$c_m(i,j+1) = \alpha_6 c_m(i,j) + \alpha_7 X(i,j) \tag{42}$$

where:

$$\alpha_2 = \frac{u\Delta t}{\Delta z} + \frac{E\Delta t}{\Delta z^2} \tag{43}$$

$$\alpha_3 = \frac{E\Delta t}{\Delta z^2} \tag{44}$$

$$\alpha_4 = 1 - \frac{2E\Delta t}{\Delta z^2} - \frac{u\Delta t}{\Delta z} - K_6 A D_L K_1 R T \Delta t/\Delta y \varepsilon \tag{45}$$

$$\alpha_5 = K_7 k_G A R T \Delta t/\varepsilon \tag{46}$$

$$\alpha_6 = 1 - K_6 D_L \Delta t/\Delta y^2 \tag{47}$$

$$\alpha_7 = K_6 P D_L K_1 \Delta t/\Delta y^2 \tag{48}$$

If $\delta_X(i,j)$ is assumed to be any bounded disturbance along the j^{th} row of the X grid and $\delta_c(i,j)$ is any bounded disturbance along the j^{th} row of the c_m grid, a sufficient condition for the output of the difference equations to be bounded is:

$$|\delta_X(i,j+1)| \leqslant |\delta_X(i,j)| \tag{49}$$

$$|\delta_c(i,j+1)| \leqslant |\delta_c(i,j)| \tag{50}$$

Using the triangle inequality, it is easily shown that eqns. (49) and (50) are satisfied for eqns. (41) and (42) if:

$$|\alpha_2| + |\alpha_3| + |\alpha_4| + |\alpha_5| \leqslant 1 \tag{51}$$

$$|\alpha_6| + |\alpha_7| \leqslant 1 \tag{52}$$

Substituting the values of α from eqns. (43) to (48) into eqns. (51) and (52), the stability criteria become:

$$\Delta t \leqslant \frac{1}{\dfrac{2E}{\Delta z^2} + \dfrac{u}{\Delta z} + \dfrac{k_G RTAD_L K_1}{\varepsilon \Delta y \left(\dfrac{D_L K_1}{\Delta y} + k_G\right)}} \tag{53}$$

$$\Delta t \leqslant \frac{\Delta y^2 \left(\dfrac{D_L K_1}{\Delta y} + k_G\right)}{D_L k_G} \tag{54}$$

It should be noted that eqn. (53) reduces to the criterion for the simple parabolic diffusion equation (*i.e.*, $\Delta t \leqslant \Delta z^2/2E$) if there is no flow ($u = 0$) and no mass transfer ($k_G = 0$).

The convergence of the numerical solution to the analytical solution as Δz is reduced is shown in Fig. 3. The analytical solution chosen was that for linear solubility with no pressure drop and no longitudinal diffusion (*cf.* FUNK AND HOUGHTON[11], equation (10)).

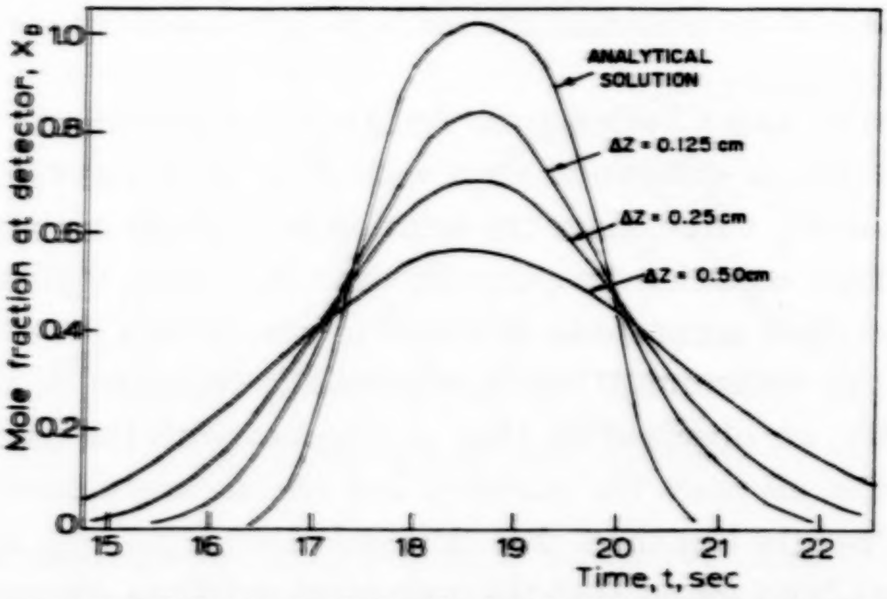

Fig. 3. Convergence of the numerical solution holding Δt constant and changing Δz.

The effect of reducing Δt is illustrated by taking the case of longitudinal dispersion in a tube with no packing governed by the equation

$$\frac{\partial X}{\partial t} + u\frac{\partial X}{\partial z} = E\frac{\partial X^2}{\partial z^2} \tag{55}$$

Subject to the initial and boundary conditions of Fig. 2, the analytical solution is easily shown to be (*cf.* BOURNIA, COULL AND HOUGHTON[17]):

$$X = \tfrac{1}{2}\,\mathrm{erf}\left(\frac{L_4 - ut}{2\sqrt{Et}}\right) + \tfrac{1}{2}\,\mathrm{erf}\left(\frac{L_1 - L_4 + ut}{2\sqrt{Et}}\right) \qquad (56)$$

The numerical solution of eqn. (55) can be obtained from the computer program merely by setting $k_G = 0$ and $\mathrm{d}P/\mathrm{d}z = 0$ in the coefficients of the difference equations (35) and (36). The effect of lowering Δt shown by Fig. 4 is to reduce the accuracy, which is the converse of what is expected. This phenomenon has been noted by REIHING AND CURLEE[18], who have suggested that there is some optimum Δt for the best accuracy. REIHING AND CURLEE point out that the difference equation for pure transport (*i.e.*, eqn. (55) in difference form with $E = 0$) is most accurate when Δt is the maximum allowable value, since the relation $u = \Delta z/\Delta t$ must also be satisfied. However, the difference equation for pure diffusion (*i.e.*, eqn. (55) in difference form with $u = 0$) becomes more accurate as Δt tends to zero within the stability criterion. In the present case the optimum Δt has been generally found to be the maximum Δt allowable for stability, an observation that also agrees with the findings of REIHING AND CURLEE. Hence in all cases the stability and the accuracy have been optimized by making Δt only slightly less than that demanded by eqns. (53) and (54). By thus maximizing Δt, it has been found that the numerical solutions are accurate enough to provide a reliable semi-quantitative picture of the effect of various parameters on the performance of gas–liquid chromatographic columns.

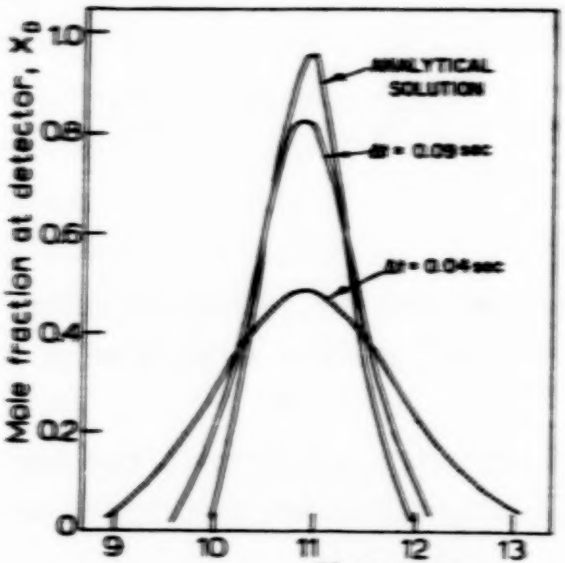

Fig. 4. Convergence of the numerical solution holding Δz constant and changing Δt.

EFFECT OF COLUMN OPERATING CONDITIONS ON THE ELUTION CURVE

The IBM-704 computer program of the equations of chromatography has been used to simulate the behavior of a typical chromatography column under various operating conditions. The system chosen was the elution of a pure isobutylene slug with an argon carrier gas using dinonyl phthalate as the stationary phase. The parameter study was made by choosing a standard set of operating conditions and then changing each of the variables one at a time, holding all other conditions constant. The standard conditions chosen were as follow:

$T = 298°\text{K}$
$P = 1 \text{ atm.}$
$A = 131.2 \text{ cm}^2/\text{cm}^3$
$u = 5 \text{ cm/sec}$
$\varepsilon = 0.40$
$h = 1.4 \cdot 10^{-4} \text{ cm}$
$D_L = 6.7 \cdot 10^{-7} \text{ cm}^2/\text{sec}$

$L_1 = 5 \text{ cm}$
$L_2 - L_1 = 1 \text{ cm}$
$L_3 - L_2 = 50 \text{ cm}$
$L_4 - L_3 = 1 \text{ cm}$
$X_0 = 1.00$
$K_1 = 0.00114$
$K_2 = 0$

Transport time without chromatography = 12.3 sec

The values of D_L and K_1 at various temperatures were taken from the data of HOUGHTON, KESTEN, FUNK AND COULL[19].

1. *Effect of gas film coefficient, k_G*

Fig. 5 shows that the peak mole fraction passes through a minimum as k_G varies in the range 0 to 10^{-3} g-mole/sec. cm².atm., while the peak time increases from the transport time with no chromatography (12.3 sec) to a maximum value of 18.5 sec when k_G is greater than 10^{-5}. The possible effect of a high gas film resistance on the shape and peak time of the elution curve has not generally been appreciated.

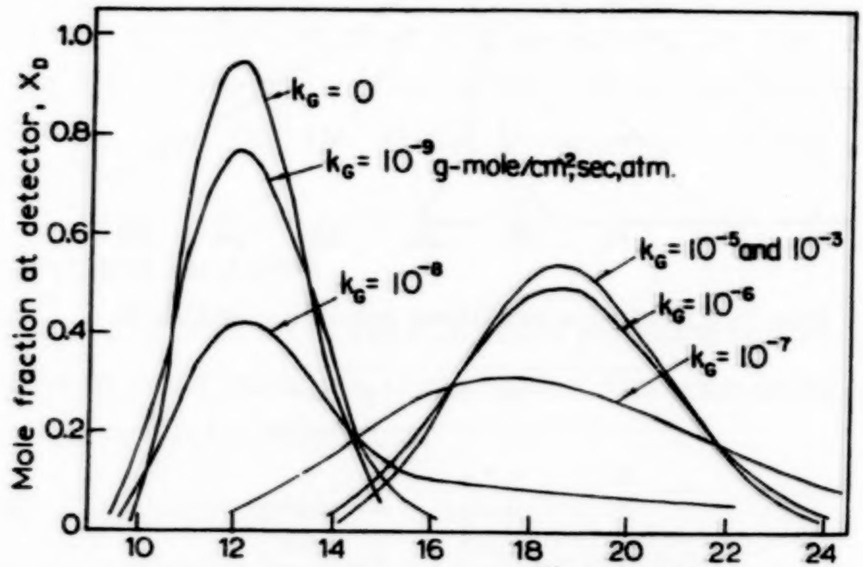

Fig. 5. Effect of gas film coefficient.

2. *Effect of longitudinal dispersion*

As E is increased in the range 0.0674 to 6.74 cm²/sec, Fig. 6 shows that the peak becomes broader, but the peak time does not change significantly. It is noted that the longitudinal dispersion coefficient E must be at least a factor of ten greater than the molecular diffusivity ($D_G = 0.0674$ cm²/sec) before the effect becomes appreciable.

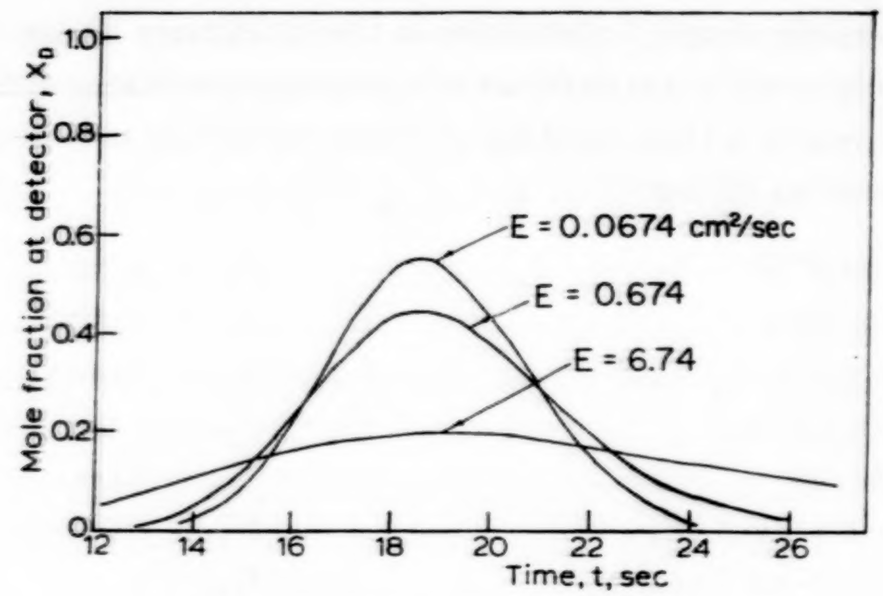

Fig. 6. Effect of longitudinal dispersion.

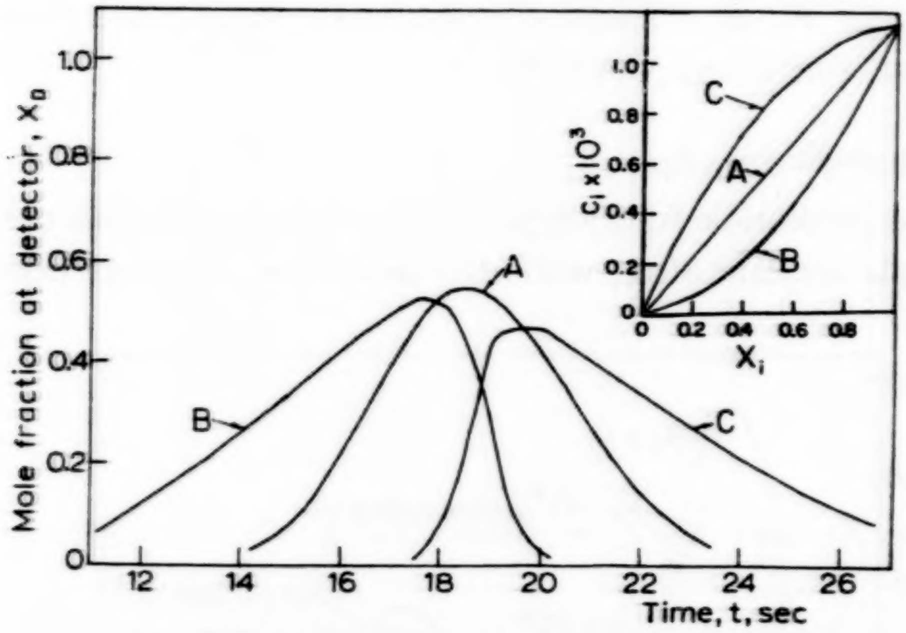

Fig. 7. Effect of a nonlinear solubility isotherm.

	$K_1 \cdot 10^3$	$K_2 \cdot 10^3$
A	1.14	0
B	0.14	+ 1.0
C	2.22	— 1.08

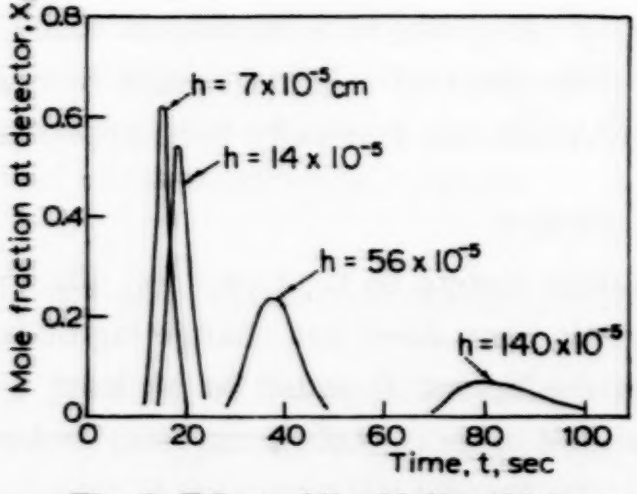

Fig. 8. Effect of liquid film thickness.

3. *Effect of a nonlinear solubility isotherm*

Fig. 7 shows that departures from ideality in the liquid phase result in considerable skewness and tailing in the elution curve with consequent displacement of the peak. The direction of the tailing is related to the type of nonlinearity as predicted by KEULEMANS[1].

4. *Effect of liquid film thickness*

As the liquid film thickness is increased, Fig. 8 shows that the curves become broader and more skewed, while the peak time is directly proportional to the film thickness. In comparing these curves it has been assumed that the depth of penetration into the liquid phase increases as the liquid film thickness increases. Although this would no doubt be true for very thin films, it would certainly not apply to very thick films which would essentially behave as semi-infinite media.

5. *Effect of temperature*

Fig. 9 shows that as temperature increases, the peak time decreases and the curves become sharper. As is found in practice, good separation must represent a compromise between peak time and band width.

6. *Effect of slug length*

The areas under the curves of Fig. 10 increase in proportion to the increase in slug length. The peak time increases because it takes longer for the centerline of a larger slug to move to the detector. The curves of Fig. 10 closely resemble those obtained by PORTER *et al.*[20] by applying the H.E.T.P. approach to the elution of slugs of varying size.

7. *Effect of total pressure*

As indicated by Fig. 11, a tenfold change in total pressure has no significant effect on either the peak time or peak mole fraction—a fact that has been verified experimentally for systems that are relatively ideal.

8. *Effect of initial slug concentration*

The curves of Fig. 12 are linear multiples of each other; that is, the curve for $X_0 = 1$ is merely four times the curves for $X_0 = 0.25$, indicating that slug concentration has no effect on the elution curve if X/X_0 is the variable.

9. *Effect of carrier gas velocity*

Fig. 13 shows that the peak time is inversely proportional to the velocity, as predicted by the analytical result of FUNK AND HOUGHTON[11]. Further, Fig. 13 shows that as the peak time increases, the area under the curve apparently increases, a fact easily explained by the following mass balance at the detector:

$$\text{Area} = \int_0^\infty X_g dt = \frac{X_0 L_s P_i}{u P_o} \tag{57}$$

Equation (57) shows that the area increases as the carrier velocity, u, decreases.

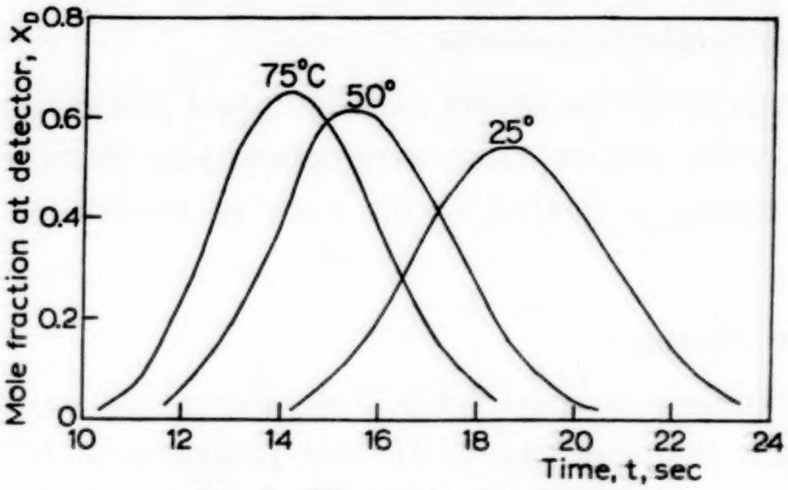

Fig. 9. Effect of temperature.

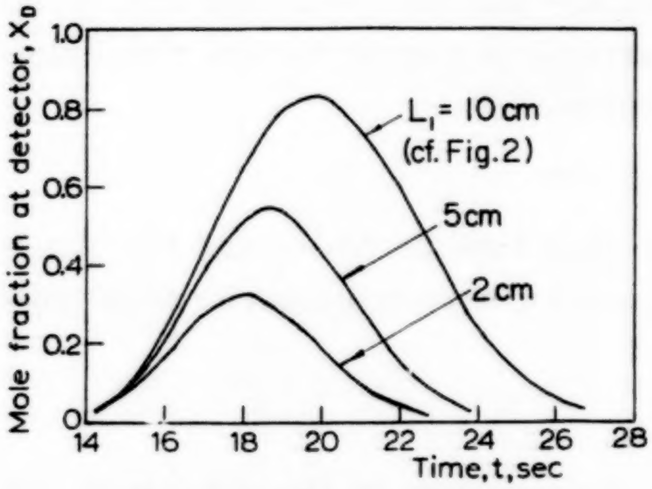

Fig. 10. Effect of slug length.

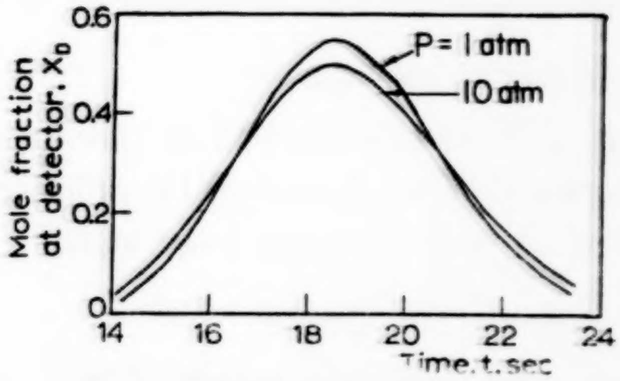

Fig. 11. Effect of total pressure.

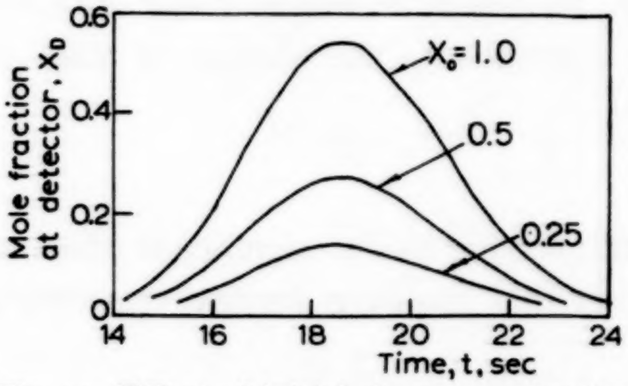

Fig. 12. Effect of initial slug concentration.

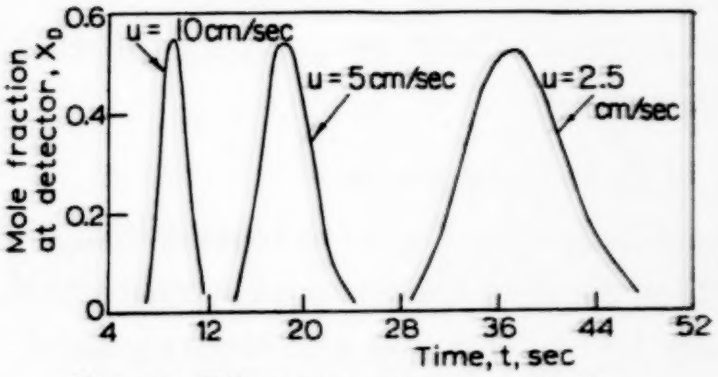

Fig. 13. Effect of carrier gas velocity.

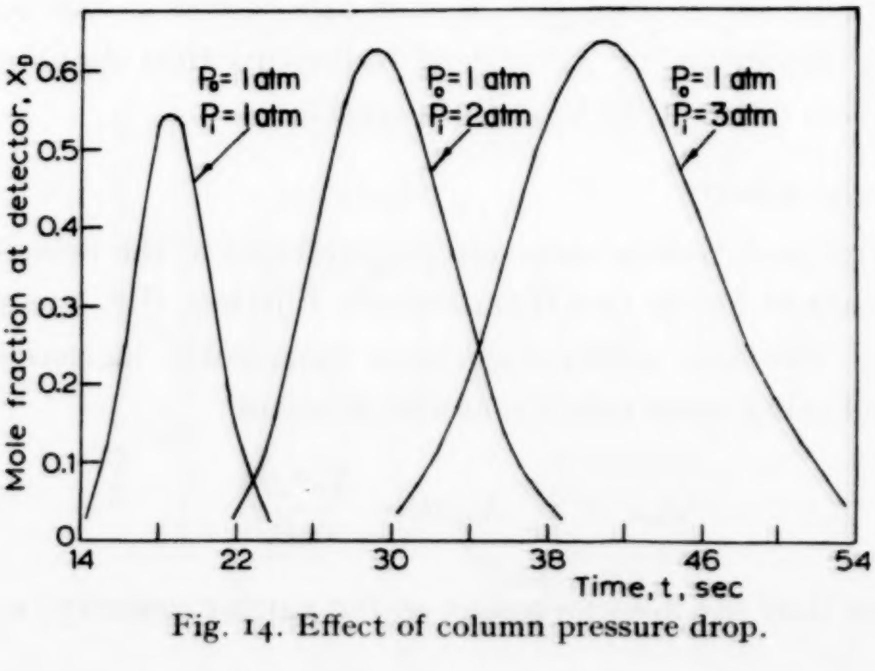

Fig. 14. Effect of column pressure drop.

10. *Effect of column pressure drop*

Fig. 14 shows that as the outlet pressure is held constant and the inlet pressure is increased, the peak time and the area under the elution curve increase. The area increases simply because a higher inlet pressure means more solute present in the slug. The peak time increases becauses the interstitial velocity decreases according to eqn. (3).

NOTATION

A = mass transfer area per unit volume of empty column, cm^2/cm^3
c = solute concentration in liquid phase, $g\text{-mole}/cm^3$
c_i = interfacial solute concentration, $g\text{-mole}/cm^3$
c_m = solute concentration at lumping point in liquid film, $g\text{-mole}/cm^3$
D_L = molecular diffusivity in liquid phase, cm^2/sec
E = longitudinal dispersion coefficient, cm^2/sec
h = liquid film thickness, cm
i = distance index in difference equations ($z = i\Delta z$)
j = time index in difference equations ($t = j\Delta t$)
k_G = gas film coefficient, $g\text{-mole}/sec.cm^2.atm.$
K = permeability of packed bed, dimensionless
K_1, K_2 = constants in nonlinear isotherm
$K_2, K_3, K_4, K_5, K_6, K_7$ = pressure-dependent parameters
L = length, cm
P = pressure, atm.
P_i = column inlet pressure, atm.
P_o = column outlet pressure, atm.
R = Universal Gas Constant, 82.06 $atm.cm^3/g\text{-mole}.\ ^\circ K$
t = time, sec
T = absolute temperature, °K
u = axial velocity through voids, cm/sec
u_i = axial velocity through voids at inlet, cm/sec
X = mole fraction of solute in vapor phase
X_D = mole fraction of solute passing the detector
X_i = mole fraction of solute at gas–liquid interface
X_0 = initial mole fraction of solute in slug
y = distance into the liquid film, cm
y_0 = a point in the liquid film, cm
z = axial distance along column, cm
$\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_6, \alpha_7$ = pressure-dependent parameters
$\beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7$ = parameters dependent upon pressure and/or pressure gradient
δ_c, δ_X = elements of a bounded set of numbers
Δ = incremental change
ε = void fraction
η = viscosity, poise

ACKNOWLEDGEMENTS

The authors wish to thank the Gulf Research and Development Company for the use of their IBM-704 digital computer and one of us (J.E.F.) is indebted to the Westinghouse Corporation for a Graduate Fellowship.

SUMMARY

The differential equations of non-ideal gas–liquid partition chromatography have been derived and simplified by introducing the concept of a lumped liquid film. The resulting equations have been solved numerically by finite difference methods using an IBM-704 digital computer. The stability and convergence of the computer solutions is discussed and the computer program is used to simulate the actual behavior of a typical column under various operating conditions. The system chosen for study was the elution of a slug of isobutylene with argon as the carrier gas and dinonyl phthalate as the stationary phase.

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SÉPARATION ET DOSAGE DU KRYPTON ET DU XÉNON PAR CHROMATOGRAPHIE EN PHASE GAZEUSE

APPLICATION AUX GAZ DE FISSION

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(Reçu le 21 décembre 1960)

INTRODUCTION

Le krypton et le xénon sont produits en quantité relativement importante lors de la fission de l'uranium. Leur formation peut entraîner des conséquences graves quant à la stabilité ou à la vie des éléments combustibles; par ailleurs ils abaissent la réactivité de la pile, et peuvent éventuellement contaminer le circuit de refroidissement s'il y a rupture de gaine ou diffusion à travers celle-ci. D'importantes recherches sont donc nécessaires pour connaître les mécanismes de diffusion de ces gaz rares à l'intérieur des solides; ces études exigent des dosages précis du krypton et du xénon généralement dilués dans des gaz usuels.

A cette fin, de nombreuses méthodes ont été utilisées; d'une part, des méthodes de comptage (chambre d'ionisation, compteur Geiger-Müller, spectromètre γ, fil chargé) d'autre part la spectrométrie de masse. Parmi ces méthodes, seule la spectrométrie de masse dispense d'utiliser des gaz très actifs et donne la quantité totale de krypton et de xénon, actifs ou non.

Cependant, la chromatographie en phase gazeuse avec détection par catharomètre permet de doser facilement les différents gaz (H_2, (O_2 + A), N_2, CO, CH_4) qui diluent en général les gaz de fission extraits des combustibles[1].

La présente étude a été effectuée dans le but de préciser les conditions d'application de la méthode chromatographique au dosage de la totalité du krypton et du xénon, sans faire appel à une technique de comptage.

BIBLIOGRAPHIE

GLUECKAUF[2] a séparé sur charbon actif un mélange de krypton et de xénon. L'élution du krypton s'effectue à —45°, et celle du xénon en faisant croître la température jusqu'à 50°.

Dans un travail plus récent[3], le même auteur a montré, en s'appuyant sur des considérations théoriques, que la séparation du krypton et du xénon sur un adsorbant était affectée par l'activité de ces gaz. En particulier l'absorption du rayonnement β ferait croître la température de l'adsorbant, ce qui diminuerait la largeur des pics ainsi que les temps de rétention. Si l'activité dans la colonne était supérieure à 14 joules/cm³, la séparation ne serait plus possible. Par contre, des gaz faiblement ou modérément actifs se comporteraient comme des gaz inertes.

GREENE[4] a réalisé de nombreuses séparations de gaz permanents en utilisant différents adsorbants. Il a, entre autres, montré qu'un mélange d'argon, de krypton, de xénon et d'oxygène pouvait être analysé à l'aide d'une colonne remplie de gel de silice thermostattée à 23° et parcourue par l'oxygène comme gaz porteur. Si ce mélange contient du méthane, la séparation doit se faire sur colonne de tamis moléculaire. L'argon, le krypton et le méthane sont séparés à 20°, le xénon en élevant la température jusqu'à 100° (thermochromatographie).

KREJČÍ, TESAŘÍK ET JANÁK[5] ont séparé un mélange de krypton, d'azote et de méthane sur une colonne contenant un mélange de charbon actif et de tamis moléculaire Linde (rapport 1/10) à 20°.

KOCH ET GRANDY[6] ont séparé un mélange de krypton et de xénon radioactifs sur colonne de charbon activé, aux températures de 0° et 20°. Le mélange de ^{85}Kr (période 10.3 années) et de ^{133}Xe (période 5.27 jours) est introduit à l'entrée de la colonne dans un courant d'hélium (débit 0.6 l/min). Une mesure de l'acitivité à la sortie de la colonne renseigne sur l'élution des deux gaz et conduit à des pics s'étalant sur plusieurs minutes. Une telle séparation avec une détection par catharomètre conduirait à une sensibilité très médiocre.

Ainsi, les travaux portant sur la séparation d'un mélange de krypton et de xénon par chromatographie en phase gazeuse sont peu nombreux. On s'est en général borné à exposer les conditions d'une bonne séparation sur divers adsorbants à différentes températures. Certains auteurs ont effectué la séparation avec des mélanges actifs, d'autres avec des mélanges inertes en opérant ou non à température constante. Mais aucun n'a décrit une méthode répondant aux trois critères suivants:

(i) Séparation à température de colonne constante, pour éviter la thermochromatographie qui rend toujours l'analyse quantitative délicate.

(ii) Méthode utilisable en présence d'autres gaz permanents (H_2, O_2, A, N_2, CO, CH_4).

(iii) Dosage quantitatif de l'ensemble des isotopes du Kr et du Xe, actifs ou non, avec une sensibilité suffisante.

RECHERCHE DES CONDITIONS D'UNE BONNE SÉPARATION

Tenant compte des impératifs précédents et des résultats antérieurs[1–6], nous avons étudié la séparation du mélange Kr–Xe sur différents adsorbants: gel de silice, charbon actif, tamis moléculaire; nous avons ensuite considéré le cas où ce mélange est dilué dans d'autres gaz.

Tous les essais ont été effectués au moyen du chromatographe Jobin et Yvon muni d'une détection par catharomètre à filaments de platine. Les colonnes ont 2 m de long et 3 mm de diamètre intérieur. L'hélium utilisé comme gaz porteur (débit

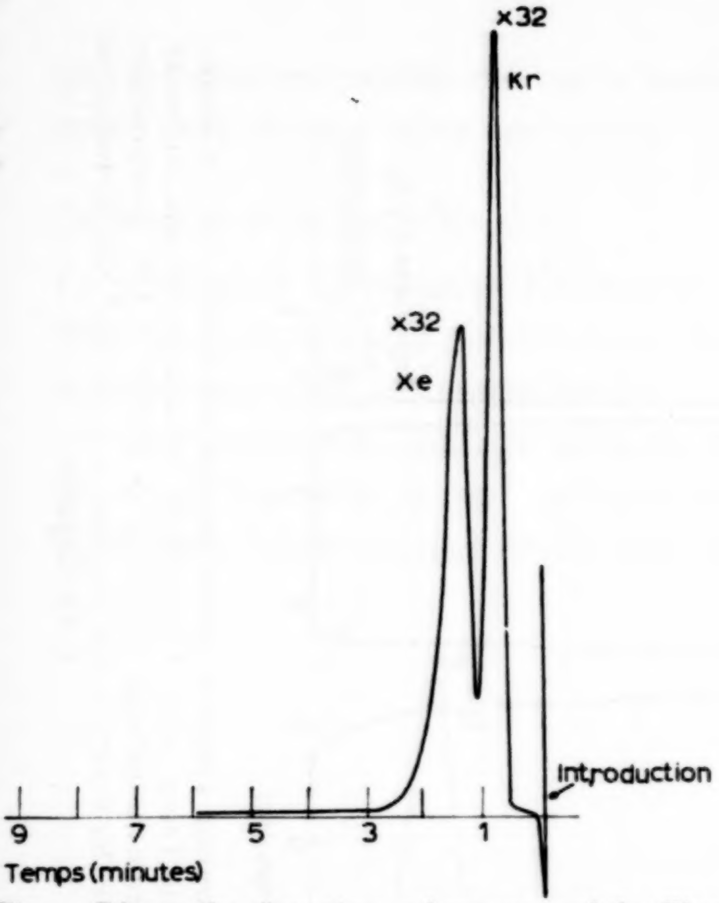

Fig. 1. Séparation krypton–xénon sur gel de silice à 50°. Colonne: gel de silice. Temp.: 50°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume du mélange introduit: 0.70 cm³ N.T.P.; composition: 0.35 cm³ Kr (Kr à 90% L'Air Liquide) + 0.35 cm³ Xe (Xe industriel L'Air Liquide).

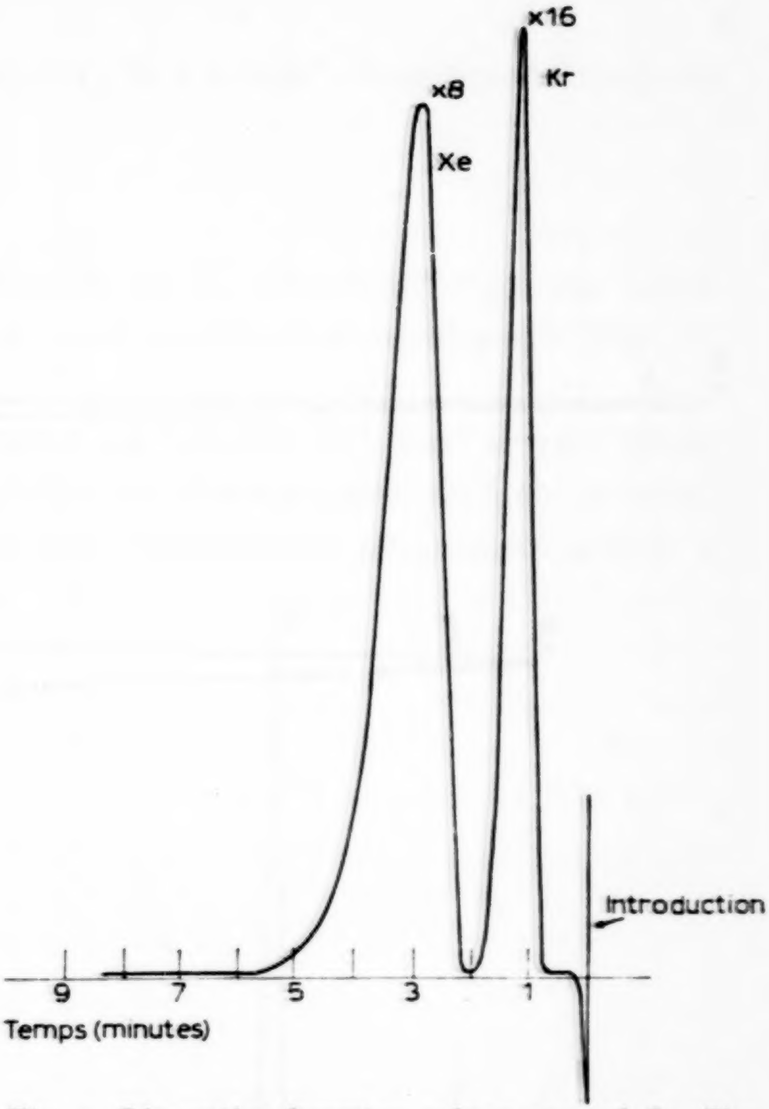

Fig. 2. Séparation krypton–xénon sur gel de silice à 20°. Colonne: gel de silice. Temp.: 20°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume du mélange introduit: 0.690 cm³ N.T.P. Composition: 0.345 cm³ Kr (Kr à 90% L'Air Liquide + 0.345 cm³ Xe (Xe industriel L'Air Liquide).

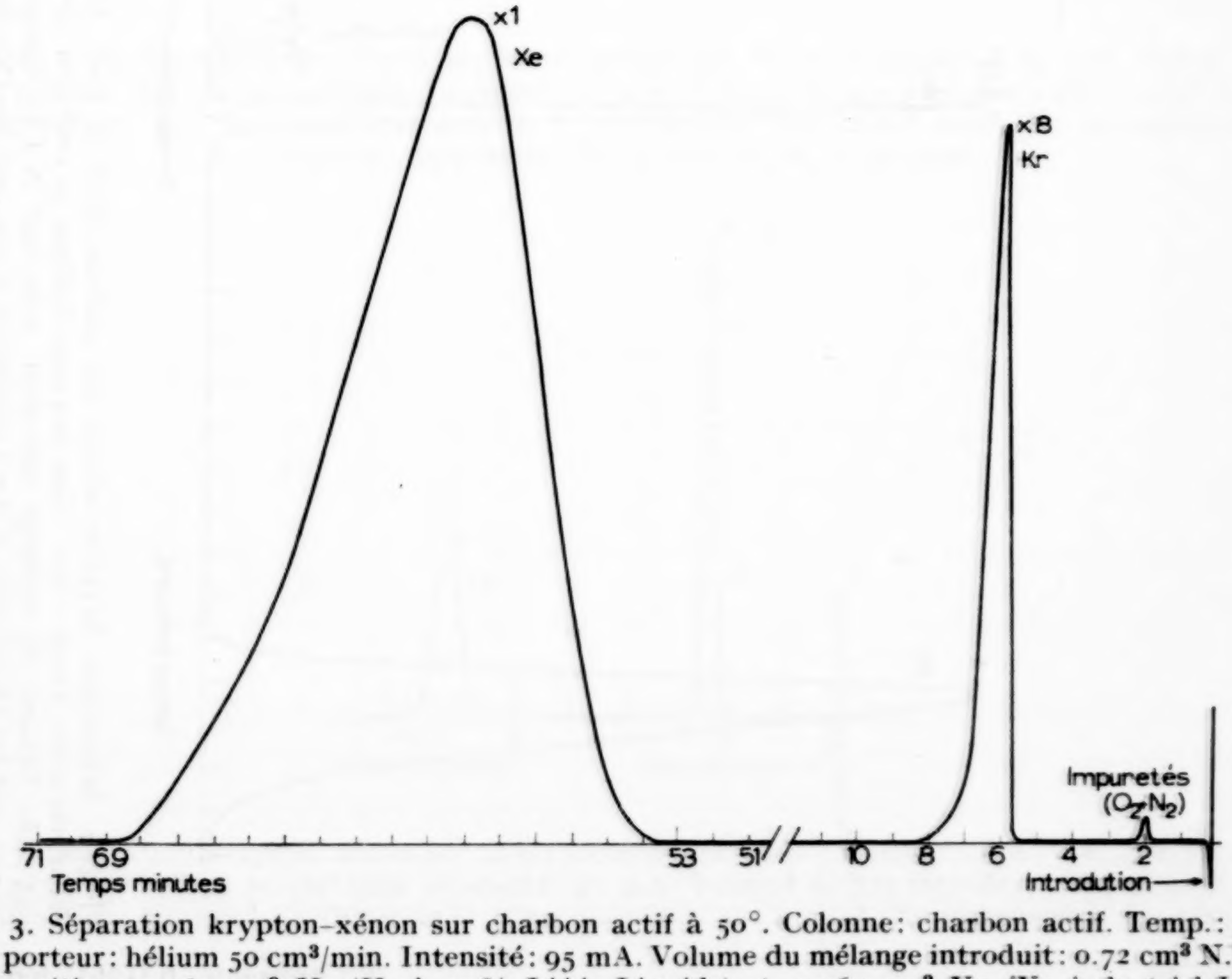

Fig. 3. Séparation krypton–xénon sur charbon actif à 50°. Colonne: charbon actif. Temp.: 50°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume du mélange introduit: 0.72 cm³ N.T.P. Composition: 0.360 cm³ Kr (Kr à 90% L'Air Liquide) + 0.360 cm³ Xe (Xe industriel L'Air Liquide).

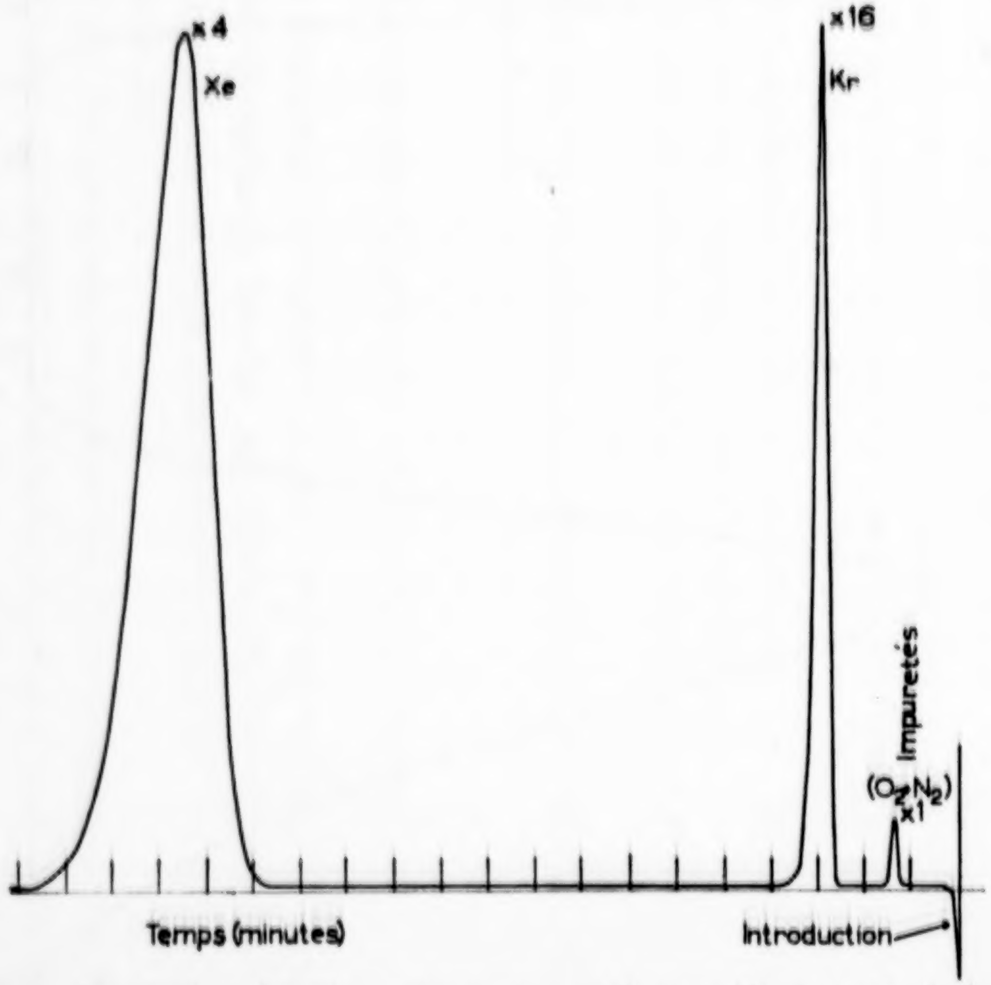

Fig. 4. Séparation krypton–xénon sur carbon actif à 100°. Colonne: charbon actif. Temp.: 100°. Gaz porteur: hélium 50 cm^3/min. Intensité: 95 mA. Volume du mélange introduit: 0.70 cm^3 N.T.P. Composition: 0.350 cm^3 Kr (Kr à 90% L'Air Liquide) + 0.350 cm^3 Xe (Xe industriel L'Air Liquide).

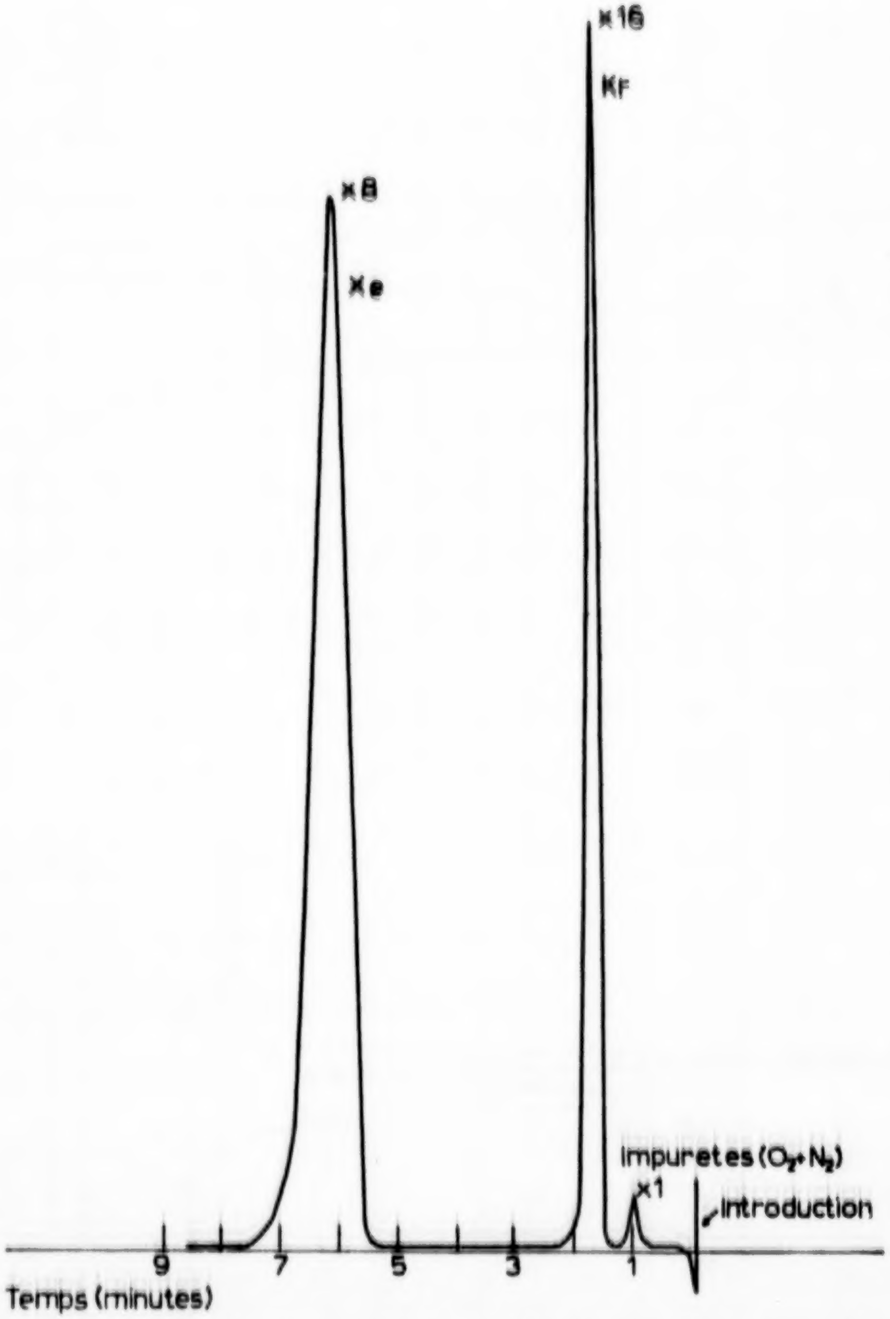

Fig. 5. Séparation krypton–xénon sur charbon actif à 150°. Colonne: charbon actif. Temp.: 150°. Gaz porteur: hélium 50 cm^3/min. Intensité: 85 mA. Volume du mélange introduit: 0.70 cm^3 N.T.P. Composition: 0.350 cm^3 Kr (Kr à 90% L'Air Liquide) + 0.350 cm^3 Xe (Xe industriel L'Air Liquide).

50 cm³/min) est purifié sur tamis moléculaire 13 X à —198°. Nous avons toujours opéré avec des gaz rares non actifs.

Séparation du mélange Kr–Xe

La séparation du mélange Kr–Xe sur colonne de gel de silice à 50° n'est pas totale (Fig. 1); par contre à 20° les deux éléments sont séparés convenablement (Fig. 2), mais les pics sont nettement étalés.

Sur colonne de charbon actif, la séparation est totale à 50°, 100° et 150° (Figs. 3, 4 et 5). Toutefois, à 150°, une bonne sensibilité est obtenue, alors qu'à 50° la sensibilité reste faible à cause de l'étalement des pics. Une colonne plus courte utilisée à

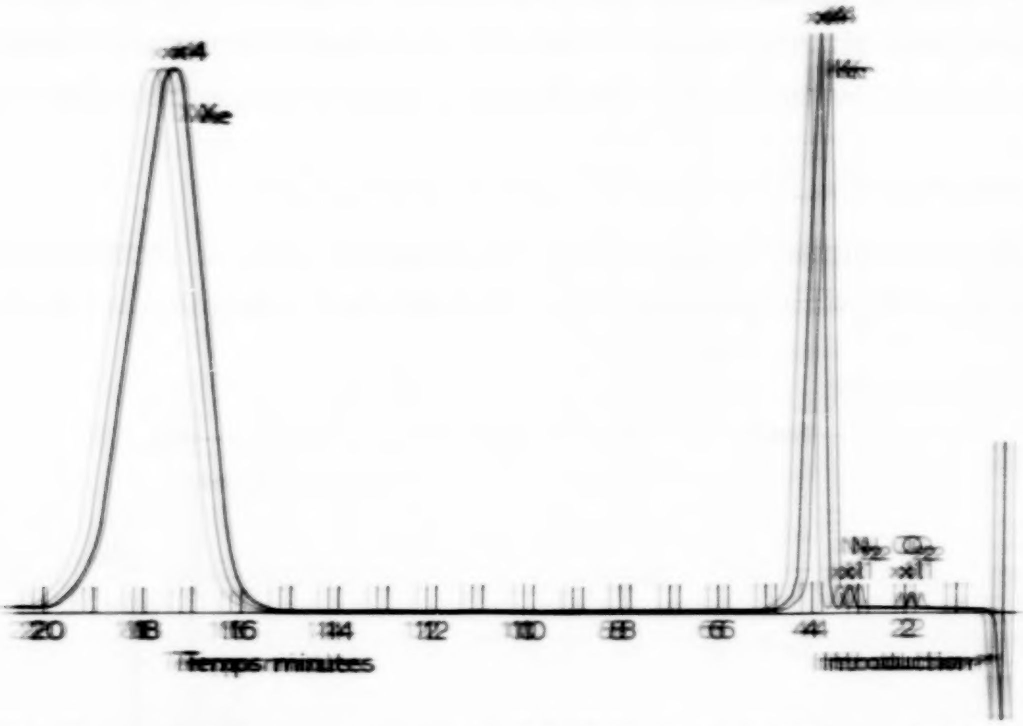

Fig. 6. Séparation krypton–xénon sur tamis moléculaire à 50°. Colonne: T.M. 5 A. Temp.: 50°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume de gaz introduit: 281.5 mm³ N.T.P. Composition: Kr: 60.7 mm³ (Kr à 90% L'Air Liquide) Xe: 220.8 mm³ (Xe industriel L'Air Liquide). Impuretés: O_2 + A: 0.22; N_2: 0.48 mm³.

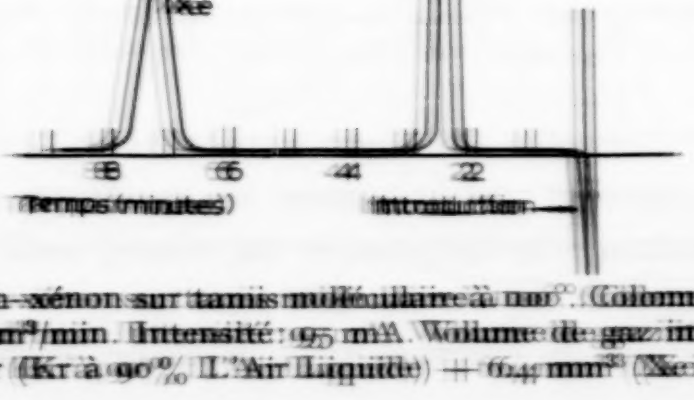

Fig. 7. Séparation krypton–xénon sur tamis moléculaire à 100°. Colonne: T.M. 5 A. Temp.: 100°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume de gaz introduit: 12.4 mm³ N.T.P. Composition: 6.0 mm³ Kr (Kr à 90% L'Air Liquide) + 6.4 mm³ (Xe industriel L'Air Liquide).

une température plus faible[5] permettrait une séparation analogue, mais les pics seraient très étalés, donc la sensibilité serait faible.

Sur colonne de tamis moléculaire 5 A, la separation du mélange krypton–xénon est très bonne à 50 comme à 100° (Figs. 6 et 7). Les pics sont très étroits, la sensibilité est très grande.

En somme, la séparation de ces deux gaz est possible sur l'un quelconque des trois adsorbants étudiés. Cependant le charbon actif et le tamis moléculaire conduisent à une meilleure sensibilité, les températures optima étant de 150° pour le charbon actif et de 100° pour le tamis moléculaire 5 A.

Dans un cas comme dans l'autre, l'utilisation d'une température plus élevée n'augmenterait pas la sensibilité. En effet, les temps de rétention seraient plus courts et les pics plus étroits, mais il faudrait diminuer l'intensité dans les filaments du catharomètre pour éviter de les détériorer à température trop élevée.

Séparation du mélange Kr–Xe–H_2–$(O_2 + A)$–N_2–CH_4–CO

Dans une étude précédente[1] nous avons pu montrer que la séparation du mélange H_2–$(O_2 + A)$–N_2–CH_4–CO pouvait être réalisée sur colonne de tamis moléculaire 5 A thermostatée à 50°, l'oxygène et l'argon sortant en un seul pic. Le gel de silice et le charbon actif ne permettent pas de séparer les constituants d'un tel mélange. Si donc on ajoute à ce mélange du krypton et du xénon, seule une colonne de tamis moléculaire est susceptible de séparer les différents gaz.

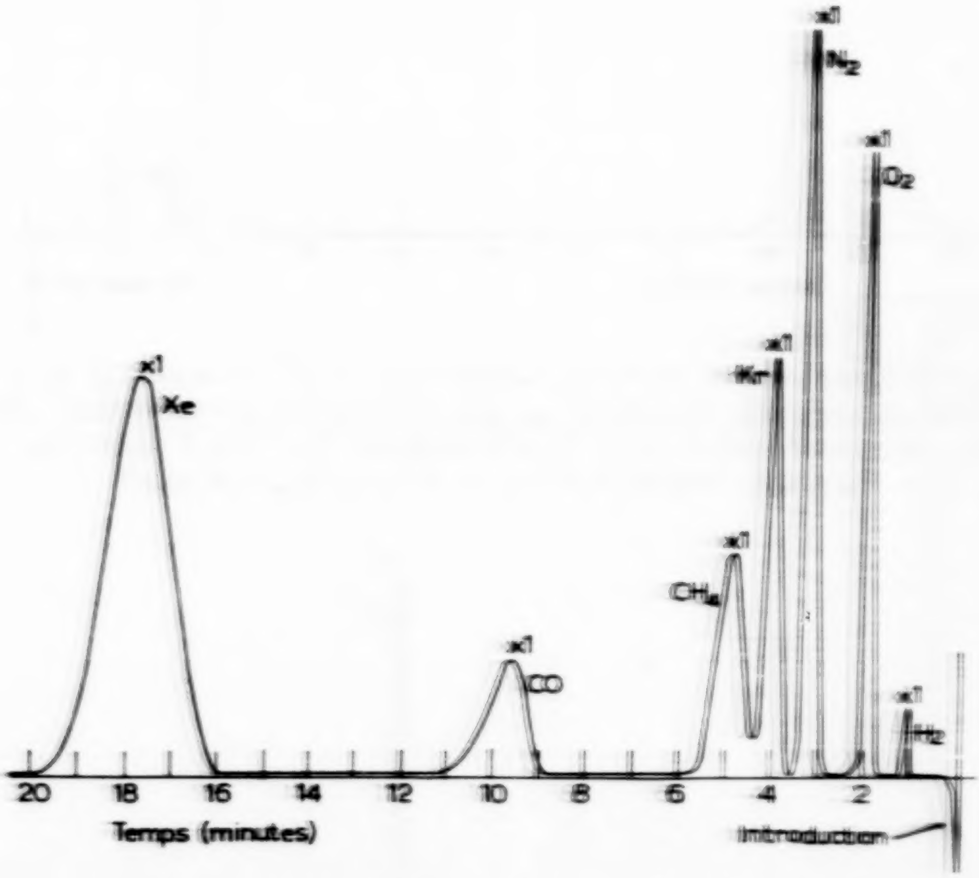

Fig. 8. Séparation sur tamis moléculaire à 50°. Colonne: T.M. 5 A. Temp.: 50°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume du mélange introduit: 182 mm³ N.T.P. Composition: H_2: 52.4; O_2: 11.0; N_2: 44.2; Kr: 11.2; CH_4: 10.8; CO: 11.5; Xe: 40.9 mm³.

A 50° (Fig. 8), les pics du krypton et du méthane se chevauchent légèrement.

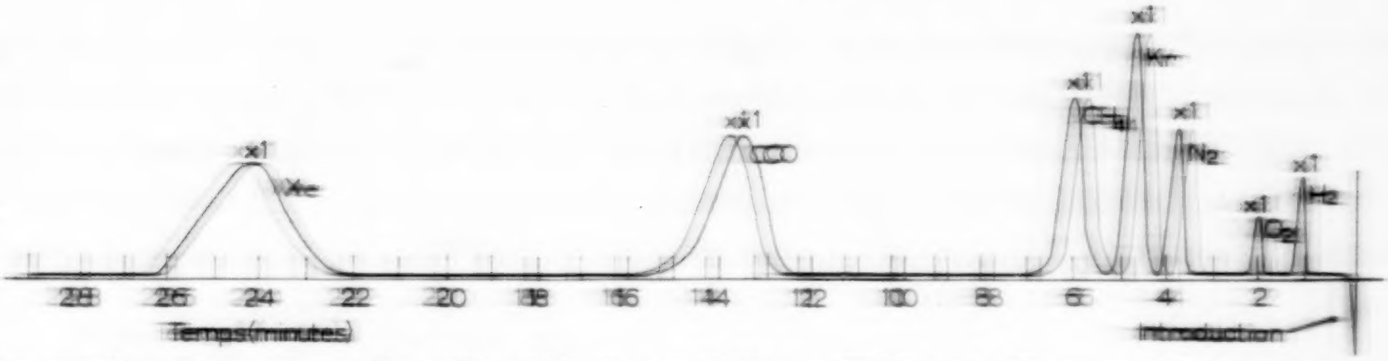

Fig. 9. Séparation sur tamis moléculaire à 40°. Colonne: T.M. 5 A. Temp.: 40°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA.

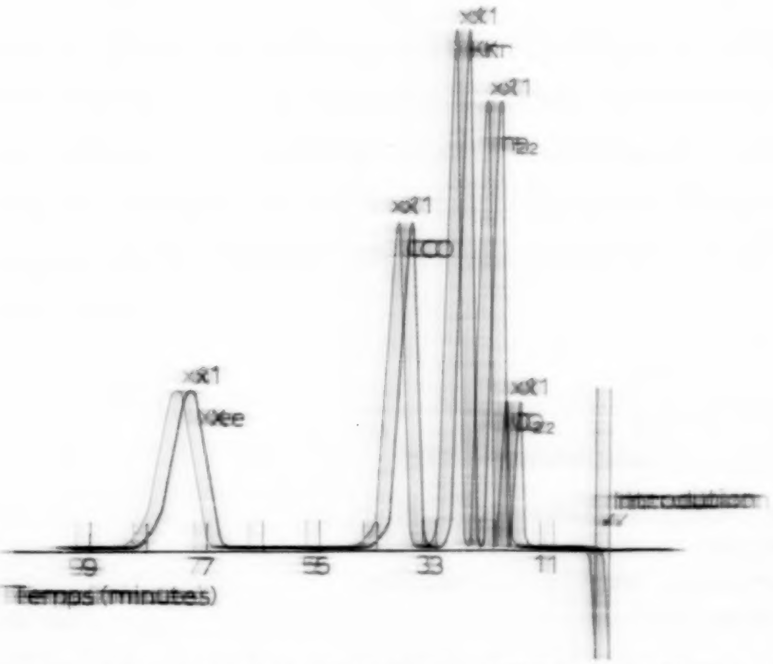

Fig. 10. Séparation sur tamis moléculaire à 100°. Colonne: T.M. 5 A. Temp.: 100°. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. Volume du mélange introduit: 1387 cm³ N.T.P. Composition O_2: 1.22; N_2: 4.88; Kr: 6.00; CO: 6.00; Xe: 6.40 mm³; He: 1360 cm³.

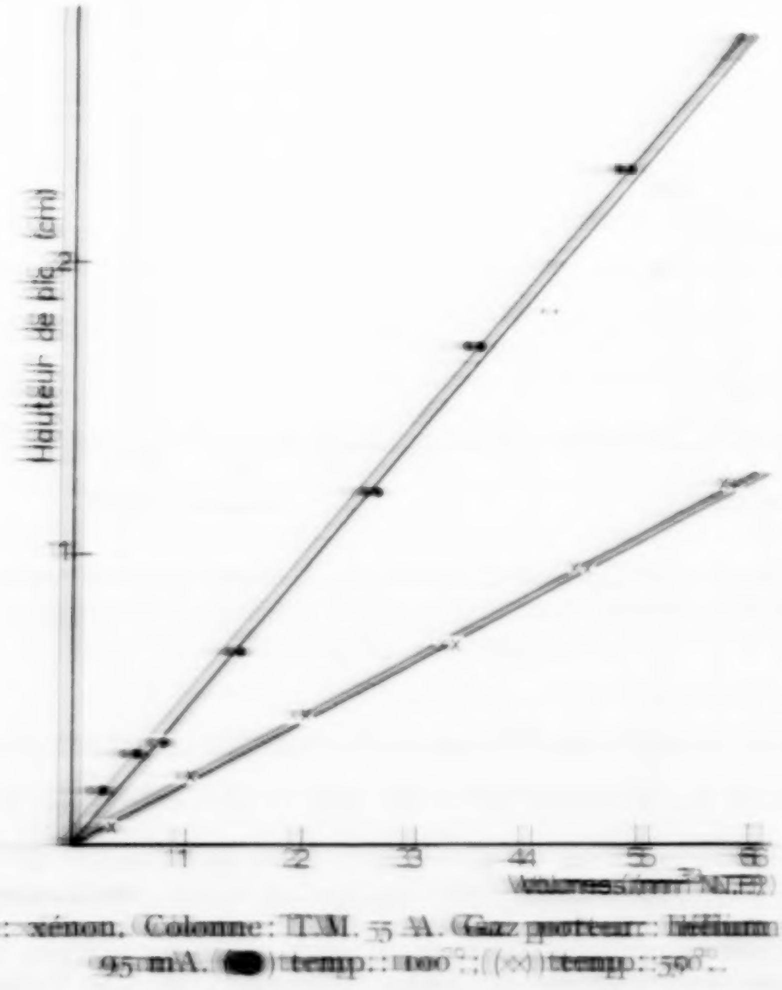

Fig. 11. Étalonnage: xénon. Colonne: T.M. 5 A. Gaz porteur: hélium 50 cm³/min. Intensité: 95 mA. (●) temp.: 100°; (×) temp.: 50°.

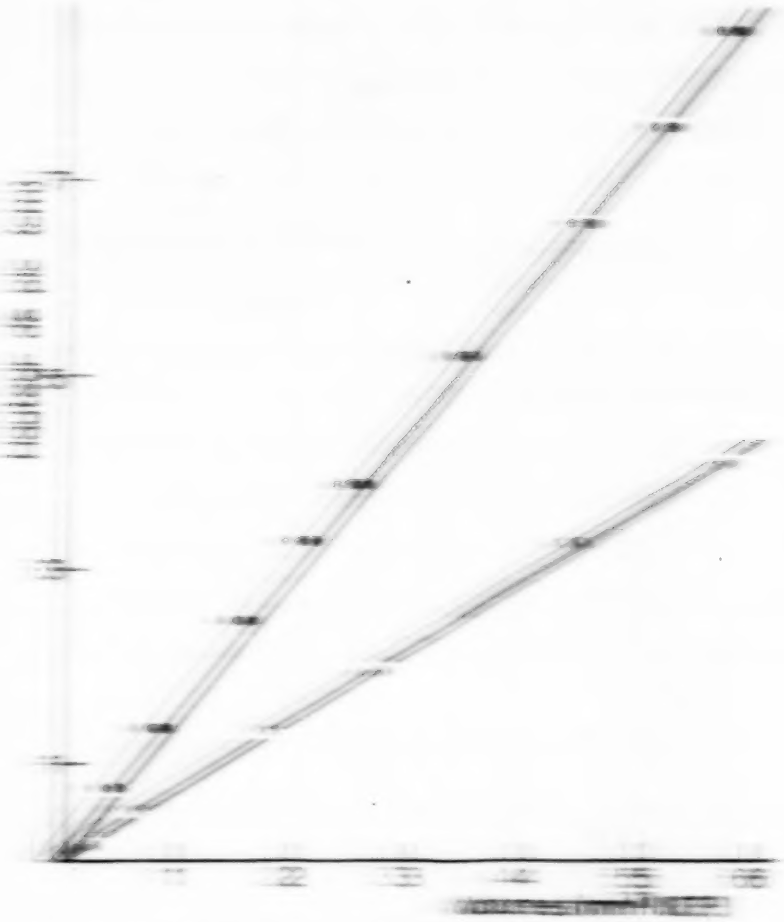

Fig. 132. Étalonnage krypton. Colonne TM 55 A. Gaz porteur : hélium 30 cm³/min. Intensité 95 mA. (●) température 100° ; (○) température 50°.

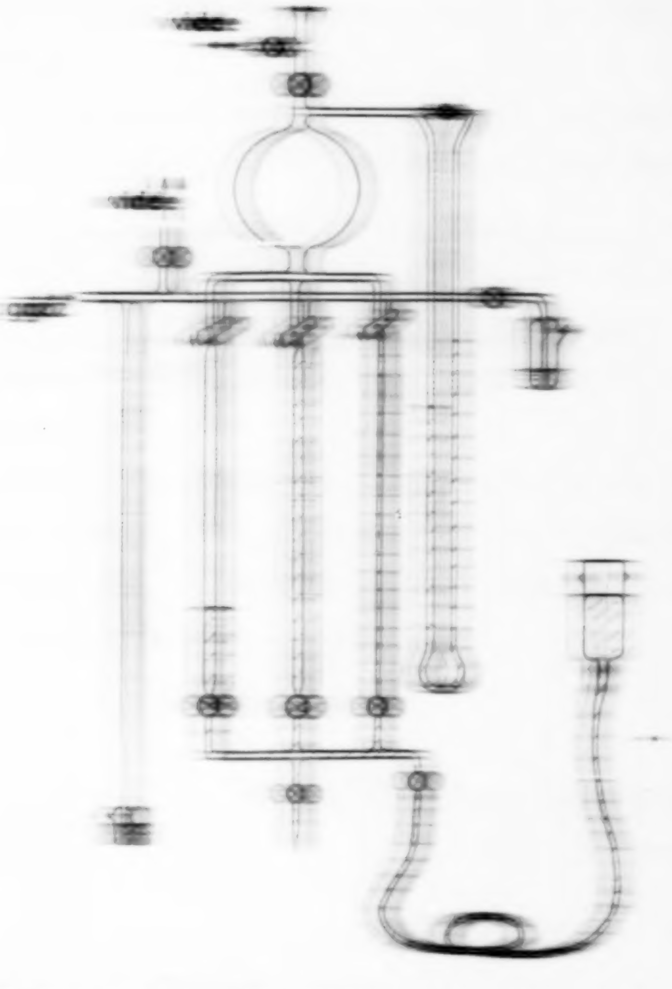

Fig. 133. Appareil utilisé pour la réalisation de mélanges gazeux.

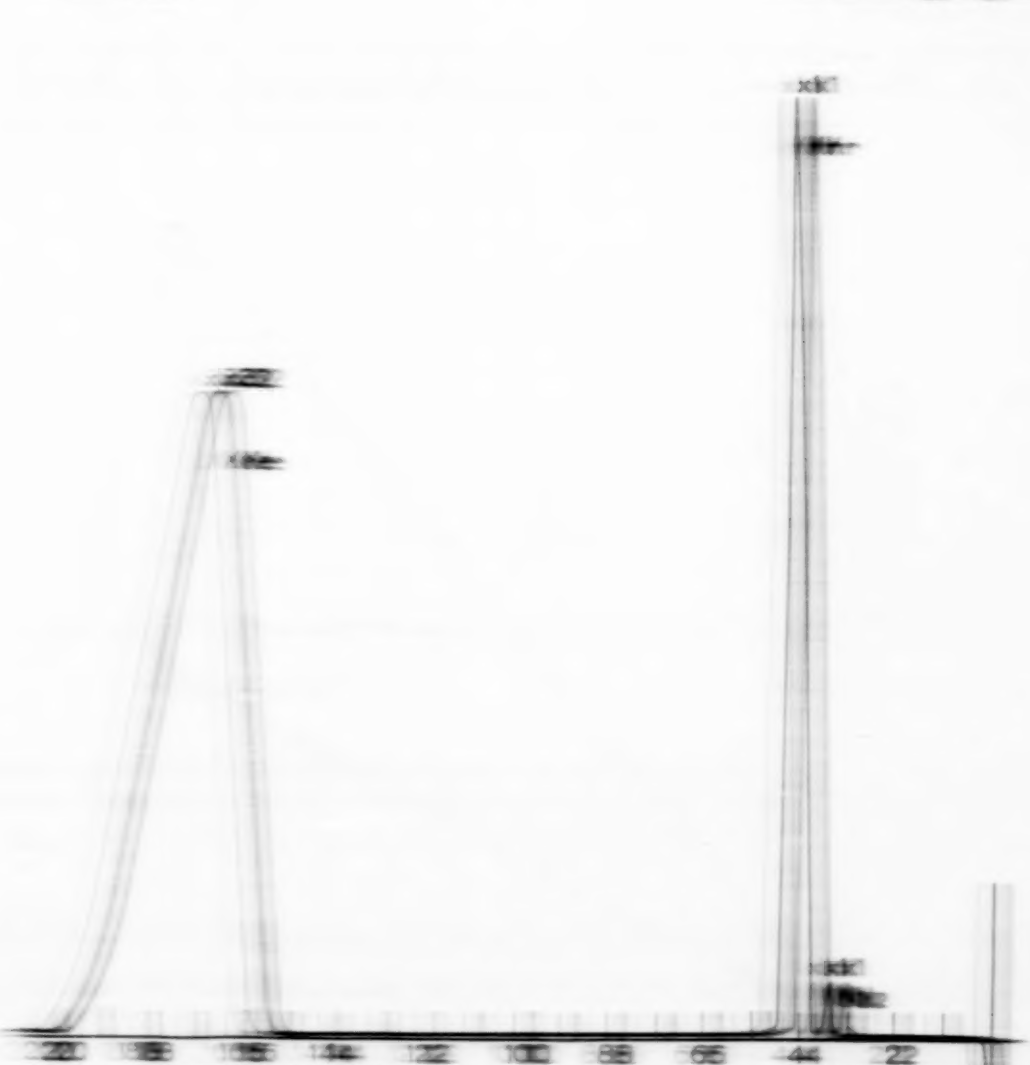

Fig. 134. Chromatogramme des impuretés du xénon. Colonne TM 55 A. Temp. : 50°. Gaz porteur : hélium 30 cm³/min. Intensité : 95 mA. Échantillon : 3 000 cm³ NTP de Xe (Xe industriel L'Air Liquide). Impuretés : N_2 0,005 %, Kr 0,007 %.

Par contre à 40° (Fig. 9) la séparation est totale, mais toutefois avec des temps de rétention très grands pour l'oxyde de carbone et surtout le xénon. Si le mélange ne contient pas de méthane, les différents constituants sont bien séparés à 100° (Fig. 10), à condition toutefois que les quantités de chacun d'eux soient faibles (< 10 mm³). A cette température, les pics sont étroits, les temps de rétention sont courts, même pour le xénon (7.3 min), et la sensibilité est grande. En présence de méthane, il convient d'opérer à 50°.

ANALYSE QUANTITATIVE

L'étalonnage est effectué à l'aide de mélanges gazeux (Figs. 8, 10, 11, 12) réalisés dans l'appareil représenté par la Fig. 13, suivant le mode opératoire décrit lors d'un travail précédent[1]. Pour obtenir les mélanges, on a simplement utilisé de l'air, de l'hydrogène, du méthane, de l'oxyde de carbone, du krypton et du xénon commerciaux. Les impuretés des gaz rares utilisés ont été déterminées (Figs. 14 et 15) et les corrections nécessaires effectuées.

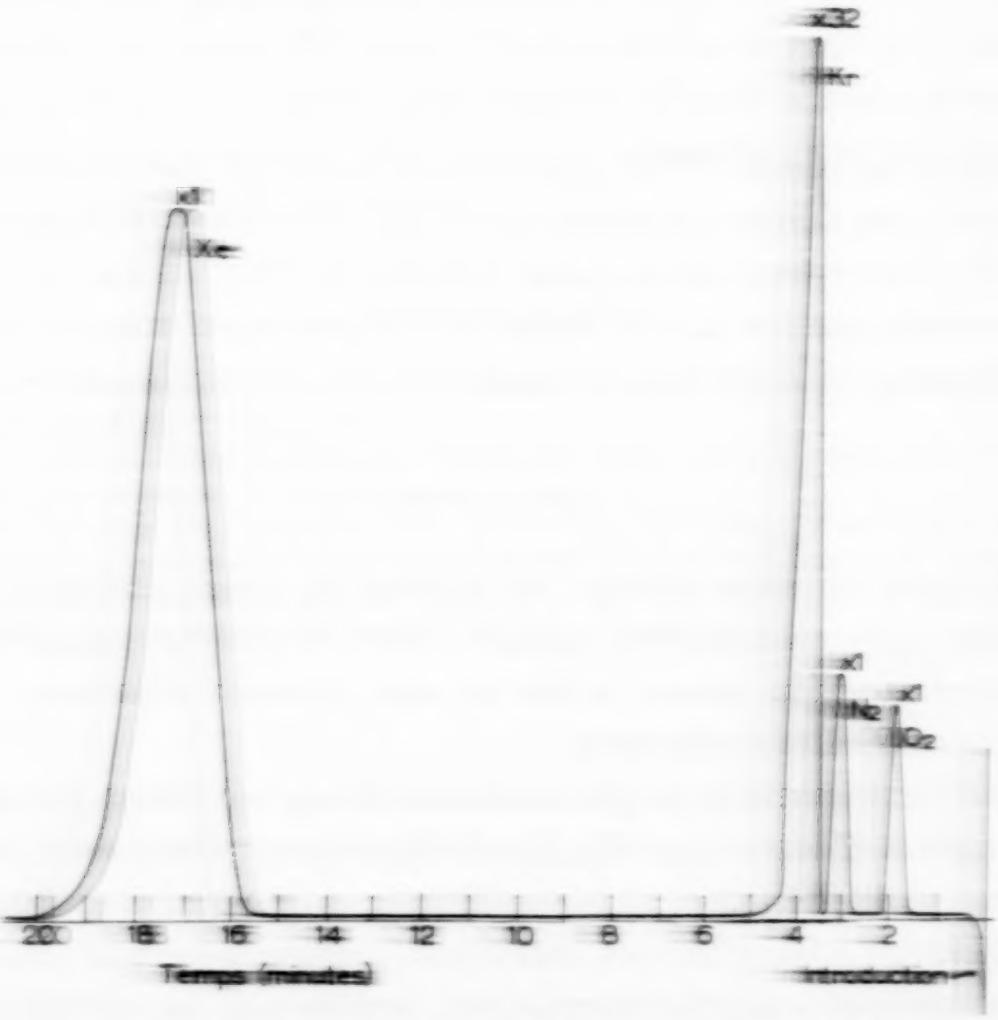

Fig. 15. Chromatogramme des impuretés du krypton. Colonne: T.M. 5 A. Temp.: 50°. Gaz porteur: hélium 50 ml/min. Intensité: 1.03 cm³ Kr (Kr à 90 % L'Air Liquide). Impuretés: A + O_2 0.30 %, N_2 0.61 %; Xe 7.04 %.

Dans le Tableau I sont indiquées les quantités de chaque constituant nécessaire pour provoquer une déviation de 1 cm sur l'enregistreur, à 50 et à 100°. La reproductibilité est de 10 à 15%, pour un pic de 2 à 3 mm de hauteur, ce qui correspond à 0.22 mm³ de krypton et 0.5 mm³ de xénon. Pour des quantités plus importantes, la reproductibilité est meilleure et la dispersion peut devenir inférieure à 5%.

Cependant, l'étalonnage [illegible] Il s'agit de savoir si la faible activité des produits de fission peut modifier ou non l'absorption sur la colonne de tamis moléculaire 5 Å. [illegible] les échantillons de combustibles quelques jours après [illegible] du krypton ^{85}Kr.

TABLEAU I

[illegible]

mm³ NTP

Gaz porteur [illegible]

[illegible]	[illegible]	[illegible]
H_2	[illegible]	
O_2	[illegible]	[illegible]
N_2	[illegible]	[illegible]
Kr	[illegible]	[illegible]
CH_4	[illegible]	[illegible]
CO	[illegible]	[illegible]
Xe	[illegible]	[illegible]

période 10,3 ans) et un xénon ^{133}Xe (période 5,3 jours). De plus, comme les quantités de gaz de fission sont faibles (quelques mm³), au bout d'un ou deux mois le ^{133}Xe aura disparu et il ne restera que la faible activité du ^{85}Kr. Dans ces conditions, on peut donc admettre que les gaz de fission se comportent comme des gaz inertes. Comme nous l'avons vu d'ailleurs [illegible] conclusion.

CONCLUSION

La chromatographie en phase gazeuse sur colonne de tamis moléculaire 5 Å munie d'une détection par catharomètre permet donc d'analyser quantitativement la totalité du krypton et du xénon [illegible] éléments combustibles irradiés, avec la sensibilité souhaitable.

L'analyse d'un échantillon de gaz contenant les gaz de fission (krypton et xénon) seuls ou mélangés à d'autres gaz (H_2, O_2, N_2, CO) est effectuée à la température de 100°. En une seule opération, tous ces éléments sont séparés et détectés avec une bonne sensibilité. Si l'échantillon est important (volume supérieur à 200 mm³) ou si du méthane est présent (cas très exceptionnel), on opère à 50° moyennant une sensibilité plus faible.

Enfin, il serait certainement possible d'améliorer encore la sensibilité de cette méthode. Cependant des quantités minimales de 0,2 mm³ de krypton et de 0,5 mm³ de xénon peuvent être dosées dès maintenant.

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[*] Le rayonnement β du ^{85}Kr [illegible] du krypton dans nos conditions opératoires [illegible] 1 mm³ de ^{85}Kr (quantité supérieure [illegible]) de chaleur de 1,6 · 10⁻⁸ cal. Il est donc [illegible] conséquence de ce phénomène.

RÉSUMÉ

La chromatographie en phase gazeuse, grâce à laquelle de nombreuses séparations ont déjà été réalisées, permet également de séparer le krypton et le xénon.

L'efficacité de plusieurs types de colonnes est étudiée à différentes températures. Seuls les tamis moléculaires permettent de doser ces gaz rares en présence des principaux gaz usuels. Au moyen de la détection par catharomètre, la totalité des différents isotopes, actifs ou non, de chacun des deux gaz peut être dosée, jusqu'à une quantité minimale de 0.2 mm³ N.T.P. pour le krypton et 0.5 mm³ pour le xénon.

SUMMARY

Gas chromatography, which has been used for the separation of numerous substances, can be applied also for the separation of krypton and xenon.

The efficiency of several types of columns was studied at various temperatures. In the presence of the principal common gases, these rare gases can only be determined with molecular sieves. By using a katharometer as detector, the total amount of the various isotopes, both active and inactive, of each of these gases can be determined. The minimum amounts that can be estimated are 0.2 mm³ N.T.P. for krypton and 0.5 mm³ N.T.P. for xenon.

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A CATALOG OF RETENTION TIMES OF A NUMBER OF ORGANIC COMPOUNDS

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(Received December 5th, 1960)

[illegible] in connection with the present study, the procedure for [illegible] pollutants, a method for the estimation of the total amount [illegible] principle of determining the optimum column conditions [illegible]. In the present study, the relative retention times of a large number [illegible] are given. Some of the compounds studied do not constitute [illegible] hazard but were included for the sake of completeness. The [illegible] been obtained on two columns and at two widely different [illegible] routine analysis the use of a large number of columns [illegible] it was decided to restrict the study to the use of a general [illegible] second, more selective, unit. After some preliminary [illegible] phosphate and β,β′-oxydipropionitrile columns were chosen as [illegible] this kind of application. Originally, two hundred compounds [illegible] amines, were studied: *tert.*-butylamine, diallylamine, [illegible] propanediamine, pyridine. Of the amines, only pyridine [illegible] columns in question. A number of compounds gave indications [illegible] on the column.

EXPERIMENTAL

[illegible] Kromo-Tog Model K-2 was used throughout the study. An [illegible] was used for introducing the liquid samples and a 1 ml gas [illegible] gaseous samples. Matheson helium was used as the carrier gas [illegible] the inlet of the column by means of a Matheson [illegible] the inlet was measured by means of a Burrell No. 340-70 [illegible]. The column was a U-shaped glass tube, 2 m long and [illegible]. The column packing was prepared in the usual way by coating a 30/60 [illegible] Chromosorb with the substrate. The weight ratio of [illegible] was 20 to 80. The column temperatures were adjusted [illegible] which were calibrated by means of a Sargeant Thermistor [illegible].

[illegible] was used as the reference solute. For any given temperature the optimum column condition was established with respect to carbon tetrachloride by varying the flow rate until maximum number of plates were obtained. Subsequently, the column condition was checked by measuring the retention time and the width of carbon tetrachloride peak.

RESULTS AND DISCUSSION

The relative retention ratios of the compounds are given in their increasing order in Tables I, II, III, and IV. The relative retention times are given at two temperatures.

TABLE I

Column condition: 20 g β,β'-oxydipropionitrile per 80 g of 30/60 mesh chromosorb (Johns-Manville).
Total weight of packing material: 12 g approx.
Column temperature: 33°.
Flow rate: 32.0 cc/min.
Detector cell temperature: 100°.
Peak characteristics of reference solute: carbon tetrachloride:
Emergence time: 35.7 min.
Peak retention time: 39.0 min.
Peak width: 0.95 min.
1 cm on the chart = 1.20 min.

Name of the compound	*B.p. °C*	*Relative retention ratio*
Propane	−42.0	0.079
Dichlorodifluoromethane (Freon 12)	−28.0	0.182
Dichlorotetrafluoroethane (Freon 114)	3.8	0.182
Propylene	−47.0	0.194
2-Methylpentane	60.0	0.208
n-Pentane	36.2	0.208
2,2-Dimethylbutane	49.7	0.209
2,3-Dimethylbutane	58.1	0.224
3-Methylbutene-1	25.0	0.224
3-Methylpentane	64.0	0.242
2,4-Dimethylpentane	80.5	0.254
n-Hexane	69.0	0.254
Pentene-2	36.4	0.254
2-Methylbutene-2	38.4	0.272
Cyclopentane	49.5	0.288
Trichlorofluoromethane (Freon 11)	24.1	0.288
3-Methylhexane	91.85	0.303
4-Methylpentene-2	58.0	0.328
2,2,4-Trimethylpentane	99.3	0.328
2,3-Dimethylpentane	89.8	0.330
Methylcyclopentane	71.8	0.330
Hexenes (mixed, 2 and 3)	64.7	0.343
Hexene-1	63.49	0.344
n-Heptane	98.4	0.360
Hexene-2	67.9	0.360
2-Methylpentene-1	61.5	0.373
Cyclohexane	81.4	0.397
Dimethylhexanes (mixed)	108–16	0.493

(*Continued on p. 222*)

TABLE I (*continued*)

Name of the compound	*B. p. °C*	*Relative retention ratio*
Ethyl ether	34.6	0.439
Heptene-3	95.8	0.463
Di-isobutylene	102.6	0.470
Isopropyl ether	67.5	0.480
2,2,5-Trimethylhexane	—	0.480
1-Chloropropene	35.0	0.484
Carbon disulfide	46.30	0.490
2,4,4-Trimethylpentene-2	101.1	0.508
n-Octane	125.8	0.510
2-Chloropropane	35.4	0.515
Methylcyclohexane	100.3	0.523
Heptene-2	98.0	0.538
tert.-Butyl ethyl ether	68.9	0.538
2,4,4-Trimethylpentene-1	101.44	0.538
tert.-Butyl chloride	51.2	0.568
Propyl isopropyl ether	51.2	0.568
tert.-Butyl methyl ether	—	0.597
Octene-1	121.3	0.688
Octene-2	121–122	0.700
2-Bromopropene	48.4	0.712
1-Chloropropane	47.2	0.716
n-Nonane	150.80	0.730
Cyclohexene	83.0	0.775
Bromoethane	38.0	0.794
Propyl ether	91.0	0.818
1-Chloro-2-methylpropane	68.9	0.853
Iodomethane	42.5	0.865
2-Bromopropane	59.6	0.882
4-Methylcyclohexene	102–3	0.925
Acetaldehyde	21.0	0.985
2-Chloro-2-methylbutane	86.0	1.01
1-Bromo-1-propene	60.2	1.02
3-Chloropropene	44.6	1.04
2-Bromo-2-methylpropane	73.03	1.06
Dimethoxymethane	44.0	1.09
1-Chlorobutane	78.0	1.17
1-Bromopropane	70.9	1.32
Cycloheptene	115.0	1.39
Iodoethane	72.2	1.41
Propionaldehyde	48.8	1.52
Isoamyl chloride	98.9	1.53
3-Chloro-2-methylpropene	72.2	1.55
2-Bromobutane	91.3	1.56
1-Bromo-2-methylpropane	91.5	1.56
Ethyl isocyanate	60.0	1.61
Dichloromethane	40.1	1.70
1,1-Dimethoxyethane	64.5	1.70
2-Iodopropane	89.5	1.70
Butyl ether	142.0	1.76
4-Vinylcyclohexene-1	130.0	1.86
Isobutyraldehyde	61.5	1.91
Diethoxymethane	89.0	1.94
3-Bromopropene	71.3	2.00
Trichloroethylene	87.0	2.045
Methyl acetate	57.1	2.10
Ethyl formate	54.3	2.18
1-Iodopropane	102.4	2.19

(*Continued on p. 223*)

TABLE I (*continued*)

Name of the compound	*B.p. °C*	*Relative retention ratio*
1,2-Dichloroethylene (*cis*)	60.1	2.20
tert.-Butyl alcohol	82.8	2.29
Methyl alcohol	64.65	2.38
Acetone	56.5	2.42
Isopropyl alcohol	82.3	2.50
Benzene	80.1	2.55
Isopropyl acetate	89.0	2.59
tert.-Butyl nitrite	63.0	2.60
Trichloromethane	61.26	2.64
Vinyl acetate	163.0	2.72
Bromotrichloromethane	104.07	2.76
2-Iodobutane	117.5	2.79
Butyraldehyde	75.7	2.80
Ethyl alcohol	78.5	2.88
Ethyl acetate	77.15	2.94
2-Fluoroethanol	103.35	3.24
Thiophene	84.12	3.70
sec.-Butyl acetate	112–13	4.03
2,3-Dichloropropene	94.0	4.15
n-Propyl acetate	101.6	4.17
sec.-Butyl alcohol	99.5	4.27
Methyl ethyl ketone	79.6	4.32
1,2-Dibromoethylene	108–10	4.44
Di-isopropyl ketone	123.7	4.60
1,2-Dichloropropane	96.8	4.67
Ethyl acrylate	99.8	4.70
Ethyl nitrate	88.7	4.88
Methyl methacrylate	100.0	5.00
Methyl isopropyl ketone	93.0	5.16
Diethyl ketone	102.7	5.17
1,2-Dichloroethane	83.5	5.50
3-Bromopropyne	88.0	5.55
1,2-Dimethoxyethane	—	5.55
Propyl alcohol	97.19	5.60
Isobutyl methyl ketone	119.0	5.73
Isopropyl nitrate	102.0	5.91
Chloropicrin	112.0	6.15
Propyl propionate	123.4	6.15
Acrylonitrile	78.0	6.29
Allyl acetate	103.4	6.45
Ethylbenzene	136.15	6.63
p-Xylene	138.35	6.63
Propyl nitrate	110.5	6.65
sec.-Isoamyl alcohol	114.0	6.65
Paraldehyde	124.4	7.83
2-Bromo-1-chloropropane	117.0	7.94
m-Xylene	139.10	7.96
Dimethyldiketone	88.0	8.60
Allyl alcohol	96–97	8.64
Cumene	152.39	8.63
Butyl alcohol	117.7	8.71
1-Bromo-2-chloroethane	107.0	10.55
o-Xylene	144.1	10.65
Ethyl carbonate	125.8	11.70
1,4-Dioxane	101.5	13.15
2-Nitropropane	120.0	14.6
1,1,2-Trichloroethane	113.5	14.8
Nitromethane	101.0	15.8
Nitroethane	114.8	18.95

TABLE II

Column condition: 20 g of β,β'-oxydipropionitrile per 80 g of 30/60 mesh chromosorb (Johns-Manville).
Total weight of packing material: 22 g (approx.).
Column temperature: 73°.
Flow rate: 48 to 54 ml/min.
Detector cell temperature: 100°.
Peak characteristics of reference solute (carbon tetrachloride):
Emergence time: 2.60 min.
Peak retention time: 2.90 min.
Peak width: 0.40 min.

Name of the compound	*B. p. °C*	*Relative retention ratio*
Propane	—42.0	0.254
3-Methylpentane	64.0	0.276
Propylene	—47.0	0.298
Dichlorodifluoromethane (Freon 12)	—28.0	0.300
2,2-Dimethylbutane	49.7	0.300
n-Pentane	36.2	0.309
2-Methylbutene-2	38.4	0.319
Dichlorotetrafluoroethane (Freon 114)	3.8	0.320
2,3-Dimethylbutane	58.1	0.340
n-Hexane	69.0	0.340
3-Methylbutene-1	25.0	0.340
2-Methylpentane	60.0	0.340
4-Methylpentene-2	58.0	0.340
Pentene-2	36.4	0.340
2,4-Dimethylpentane	80.5	0.361
3-Methylhexane	91.85	0.362
n-Heptane	98.4	0.333
Methylcyclopentane	71.8	0.383
Cyclopentene	49.5	0.404
2,2,4-Trimethylpentane	99.3	0.404
2,3-Dimethylpentane	89.8	0.425
Dimethylhexanes (mixed)	108–16	0.446
2-Methylpentene-1	61.5	0.448
Hexenes (mixed, 2 and 3)	64.67	0.448
Hexene-1	63.49	0.470
Hexene-2	67.9	0.470
Trichlorofluoromethane (Freon 11)	24.1	0.470
Ethyl ether	34.6	0.480
Isopropyl ether	67.5	0.500
Cyclohexane	81.4	0.510
Di-isobutylene	102.6	0.510
Heptene-3	95.8	0.510
2,2,5-Trimethylhexane	—	0.510
1-Chloropropane	47.2	0.522
tert.-Butyl ethyl ether	68.9	0.553
1-Chloropropene	35.0	0.553
Methylcyclohexane	100.3	0.553
n-Octane	125.8	0.553
2,4,4-Trimethylpentene-2	101.1	0.553
Heptene-2	98.0	0.574
Carbon disulfide	46.3	0.575
2,4,4-Trimethylpentene-1	101.44	0.595
2-Chloropropane	35.4	0.605
tert.-Butyl methyl ether	—	0.660
tert.-Butyl nitrite	63.0	0.723
2-Bromopropene	48.4	0.760

(Continued on p. 225)

TABLE II (*continued*)

Name of the compound	*B.p. °C*	*Relative retention ratio*
Propyl ether	91.0	0.760
Octene-1	121.3	0.805
n-Nonane	150.8	0.810
Cyclohexene	83.0	0.850
Octene-2	121–22	0.850
Bromoethane	38.0	0.865
1-Chloro-2-methylpropane	68.9	0.915
Methylthiomethane	37.5–38	0.915
Iodomethane	42.5	0.930
4-Methylcyclohexene	102–3	0.950
2-Bromopropane	59.6	1.000
1-Bromo-1-propene	60.2	1.02
2-Bromo-2-methylpropane	73.03	1.04
Dimethoxymethane	44.0	1.06
3-Chloropropene	44.6	1.08
2-Chloro-2-methylbutane	86.0	1.12
1-Chlorobutane	78.0	1.16
Cycloheptene	115.0	1.21
1-Bromopropane	70.9	1.30
Iodoethane	72.2	1.40
1-Chloro-3-methylbutane	98.9	1.49
1,1-Dimethoxyethane	64.5	1.49
3-Chloro-2-methylpropene	72.2	1.49
1-Bromo-2-methylpropane	91.5	1.52
2-Bromobutane	91.3	1.57
Butyl ether	142.0	1.62
Dichloromethane	40.1	1.62
2-Iodopropane	89.5	1.63
Propionaldehyde	48.8	1.70
Ethyl isocyanate	60.0	1.74
Diethoxymethane	89.0	1.78
Ethyl formate	54.3	1.78
1-Chloropentane	108.2	1.88
Isobutyraldehyde	61.5	1.89
1,2-Dichloroethylene (*cis*)	60.1	1.91
Trichloroethylene	87.0	1.92
Methyl acetate	57.1	1.95
1-Bromobutane	101.6	2.00
tert.-Butyl alcohol	82.8	2.04
4-Vinylcyclohexene-1	130.0	2.06
3-Bromopropene	71.3	2.10
Vinyl acetate	163.0	2.19
1-Iodopropane	102.4	2.20
Trichloromethane	61.26	2.28
Methyl alcohol	64.65	2.30
Benzene	80.1	2.34
Isopropyl acetate	89.0	2.35
Isopropyl alcohol	82.3	2.40
Ethyl acetate	77.15	2.48
Ethyl alcohol	78.5	2.54
2-Iodobutane	117.5	2.65
Acetone	65.5	2.68
Butyraldehyde	75.7	2.76
Bromotrichloromethane	104.07	2.78
Ethyl acrylate	99.8	3.24
Methyl methacrylate	100.0	3.34
Thiophene	84.12	3.39

(*Continued on p. 226*)

TABLE II (*continued*)

Name of the compound	*B. p. °C*	*Relative retention ratio*
Isopropyl nitrate	102.0	3.53
sec.-Butyl acetate	112–13	3.82
sec.-Butyl alcohol	99.5–100	3.83
sec.-Isoamyl alcohol	114.0	3.94
Methyl ethyl ketone	79.6	4.00
n-Propyl acetate	101.6	4.00
1,2-Dibromoethylene	108–10	4.05
Trichloroacetaldehyde	98.0	4.20
2,3-Dichloropropene	94.0	4.21
Ethyl nitrate	88.7	4.39
Di-isopropyl ketone	123.7	4.49
1,2-Dichloropropane	96.8	4.55
Methyl isopropyl ketone	93.0	4.60
Allyl alcohol	96–7	4.85
1,2-Dichloroethane	83.5	4.85
1,2-Dimethoxyethane	—	4.85
2-Fluoroethanol	103.35	4.87
Propyl propionate	123.4	4.90
Allyl acetate	103.4	5.05
Acrylonitrile	78.0	5.23
Ethylbenzene	136.15	5.28
Butyl alcohol	117.7	5.33
3-Bromopropyne	88.0	5.40
Propyl nitrate	110.5	5.60
Chloropicrin	112.0	5.64
Dimethyldiketone	88.0	5.75
Paraldehyde	124.4	6.23
Butyl acetate	126.5	6.27
p-Xylene	138.35	6.55
2-Bromo-1-chloropropane	117.0	6.64
Isobutyl methyl ketone	119.0	6.65
Cumene	152.39	7.28
Ethane nitrile	82.00	7.35
Butyl nitrite	75.0	7.50
Isobutyronitrile	107.0	7.53
1-Bromo-2-chloroethane	107.0	7.65
1,4-Dioxane	101.5	7.78
o-Xylene	144.41	8.23
Propane nitrile	97.10	9.10
Amyl nitrite	—	9.20
Ethyl carbonate	125.8	9.63
Isoamyl alcohol	130.5	9.75
1,1,2-Trichloroethane	113.5	11.6
Pyridine	115.3	13.0
Nitromethane	101.0	14.25
2-Nitropropane	120.0	15.1
Nitroethane	114.8	18.0

TABLE III

Column condition: 20 g of tri-*m*-tolyl phosphate per 80 g of 30/60 mesh chromosorb.
Total weight of packing material: 22 g (approx.).
Column temperature: 93°.
Flow rate: 49–54 ml/min.
Detector cell temperature: 150°.
Peak characteristics of reference solute (carbon tetrachloride):
  Emergence time: 4.0 min.
  Peak retention time: 4.3 min.
  Peak width: 0.60 min.

Name of the compound	*B. p. °C*	*Relative retention ratio*
n-Pentane	36.2	0.219
2,2-Dimethylbutane	49.7	0.219
Isopentane	28.0	0.220
Propane	—42.17	0.232
Trichlorofluoromethane (Freon 11)	24.1	0.233
Propylene	—47.0	0.246
Pentene-2	36.4	0.247
2-Methylbutene-2	38.4	0.267
Dichlorodifluoromethane (Freon 12)	—28.0	0.274
Dichlorotetrafluoroethane (Freon 114)	3.8	0.274
2,3-Dimethylbutane	58.1	0.274
2-Methylpentane	60.0	0.301
Ethyl ether	34.6	0.310
3-Methylbutene-1	25.0	0.328
n-Hexane	69.0	0.329
Cyclopentane	49.5	0.356
3-Methylpentane	64.0	0.360
Hexene-1	63.48	0.363
2,4-Dimethylpentane	80.5	0.370
4-Methylpentene-2	58.0	0.373
1-Chloropropene	35.0	0.384
2-Chloropropane	35.4	0.390
Hexenes (mixed, 2 and 3)	64.67	0.410
2-Methylpentene-1	61.5	0.410
tert.-Butyl methyl ether	—	0.425
Dimethoxymethane	44.0	0.425
Hexene-2	67.9	0.438
Isopropyl ether	67.5	0.460
Methylcyclopentane	71.8	0.467
Methylthiomethane	37.5–38	0.485
Methyl alcohol	64.65	0.500
2,3-Dimethylpentane	89.7	0.507
Bromoethane	38.0	0.508
tert.-Butyl ethyl ether	68.9	0.520
Carbon disulfide	46.3	0.520
1-Chloropropane	47.2	0.520
2,2,4-Trimethylpentane	99.3	0.520
3-Methylhexane	91.85	0.530
Isopropenyl bromide	48.4	0.548
3-Chloropropene	45.6	0.550
Iodomethane	42.5	0.550
Ethyl formate	84.3	0.585
n-Heptane	98.4	0.589
Propyl isopropyl ether	82.0–83	0.590
Methyl acetate	57.1	0.600
1,2-Dichloroethylene (*trans*)	48.4	0.615
Propionaldehyde	48.8	0.618

(Continued on p. 228)

TABLE III (*continued*)

Name of the compound	*B. p. °C*	*Relative retention ratio*
Acetone	56.5	0.630
Cyclohexane	81.4	0.630
1,1-Dimethoxyethane	64.5	0.657
2-Bromopropane	59.6	0.660
Dichloromethane	40.1	0.660
Cyclohexane epimethylene oxide	—	0.671
Isobutyraldehyde	61.5	0.685
Dimethylhexanes (mixed)	108–16	0.696
Heptene-3	95.8	0.699
Ethyl isocyanate	60.0	0.712
1-Bromo-1-propene	60.2	0.720
Ethyl alcohol	78.5	0.720
Butyl nitrite	75.0	0.726
1-Chloro-2-methylpropane	68.9	0.730
Di-isobutylene	102.6	0.740
2,4,4-Trimethylpentene-1	101.44	0.740
tert.-Butyl alcohol	82.8	0.760
Isopropyl alcohol	82.3	0.765
tert.-Butyl nitrite	63.0	0.767
Vinyl acetate	72.3	0.770
Propyl ether	91.0	0.775
2-Bromo-2-methylpropane	73.3	0.800
Heptene-2	98.0	0.808
2,4,4-Trimethylpentene-2	101.1	0.810
2,2,5-Trimethylhexane	—	0.850
Cyclohexene	83.0	0.876
Ethyl acetate	77.15	0.880
Iodoethane	72.2	0.905
1-Bromopropane	70.9	0.920
2-Chloro-2-methylbutane	86.0	0.930
1-Chlorobutane	78.0	0.955
2-Iodopropane	89.5	0.985
Butyraldehyde	75.7	1.01
Diethoxymethane	89.0	1.04
Ethane nitrile	82.0	1.04
n-Octane	125.8	1.04
3-Chloro-2-methylpropene	72.2	1.04
Isopropyl acetate	89.0	1.06
Acrylonitrile	78–9	1.09
Methylcylohexane	100.3	1.12
Methyl ethyl ketone	79.6	1.12
1,2-Dichloroethylene (*cis*)	60.1	1.15
4-Methylcyclohexane	102–3	1.17
Octene-1	121.3	1.23
Benzene	80.1	1.24
1-Bromo-2-methylpropane	91.5	1.28
3-Bromopropene	71.3	1.28
Trichloromethane	61.26	1.28
Propyl alcohol	97.19	1.29
Dimethyldiketone	88.00	1.31
2-Bromobutane	91.3	1.33
1-Chloro-3-methylbutane	98.9	1.34
Ethyl nitrate	88.7	1.37
Octene-2	121–2	1.39
sec.-Butyl alcohol	99.5–100	1.43
Allyl alcohol	96–7	1.45
1,2-Dimethoxyethane	—	1.46

(*Continued on p. 229*)

TABLE III (*continued*)

Name of the compound	*B. p. °C*	*Relative retention ratio*
Methyl isopropyl ketone	93.0	1.50
Trichloroethylene	87.0	1.52
n-Propylacetate	101.6	1.56
Propane nitrile	97.1	1.62
Thiophene	84.12	1.62
1,2-Dichloroethane	83.5	1.63
1-Bromobutane	101.6	1.65
Allyl acetate	103–4	1.76
Ethyl acrylate	99.8	1.78
Methyl methacrylate	100.0	1.78
1-Iodopropane	102.4	1.82
Isopropyl nitrate	102.0	1.83
Nitromethane	101.0	1.84
Cycloheptene	115.0	1.84
Diethyl ketone	102.7	1.85
1-Chloropentane	108.2	1.86
Isobutyronitrile	107–8	1.86
3-Bromopropyne	88–90	1.89
2-Fluoroethanol	103.35	1.89
n-Nonane	150.8	1.93
sec.-Butyl acetate	112–13	1.94
Butyl formate	106.8	2.17
1,2-Dichloropropane	96.8	2.04
1,4-Dioxane	101.5	2.20
Crotonaldehyde	104–5	2.22
2,3-Dichloropropene	94.0	2.30
Isobutyl methyl ketone	119.0	2.32
Trichloroacetaldehyde	98.0	2.32
1,2-Dibromoethylene (*trans*)	108.0	2.52
Butyl ether	142.0	2.53
2-Iodobutane	117.5	2.55
Vinylcyclohexene-1	130.0	2.58
sec.-Isoamyl alcohol	114.0	2.60
Butyl alcohol	117.7	2.62
Propyl propionate	123.4	2.62
Propyl nitrate	110.5	2.63
Nitroethane	114.80	2.71
Di-isopropyl ketone	123.70	2.74
Butane nitrile	118.0	2.90
Tetrachloroethylene	121.02	2.90
1-Bromo-2-chloroethane	107.0	3.10
Paraldehyde	124.4	3.10
Butyl acetate	126.5	3.22
2-Nitropropane	120.0	3.25
2-Propyn-1-ol	114–5	3.31
Ethyl carbonate	125.8	3.43
Chloropicrin	112.0	3.51
3-Butane nitrile	116–9	3.53
Epichlorohydrin	117.0	3.60
n-Decane	174.0	3.70
2-Bromo-1-chloropropane	117.0	3.83
Pyridine	115.3	4.16
1,2-Dibromoethylene (*cis*)	110.0	4.35
Ethylbenzene	136.15	4.36
Isoamyl alcohol	130.5	4.40
p-Xylene	138.35	4.52
1,1,2-Trichloroethane	113.5	4.75

(*Continued on p. 230*)

TABLE III (*continued*)

Name of the compound	*B.p. °C*	*Relative retention ratio*
m-Xylene	139.1	4.75
Isoamyl acetate	142.5	4.86
n-Amyl alcohol	138.0	4.94
2,4-Pentanedione	139.0	5.68
o-Xylene	144.41	6.11
Amyl acetate	148.0	6.19
Cumene	152.39	6.70
Octyl alcohol	195.0	8.15
Cyclohexanone	156.7	10.77
Bromocyclohexane	163–65	11.79
Decahydronaphthalene	194.6	13.46

TABLE IV

Column condition: 20 g of tri-*m*-tolyl phosphate per 80 g of 30/60 mesh chromosorb.
Total weight of packing material: 22 g (approx.).
Column temperature: 113°.
Flow rate: 56–60 ml/min.
Detector cell temperature: 150°.
Peak characteristics of reference solute (carbon tetrachloride):
Emergence time: 1.70 min.
Peak retention time: 1.90 min.
Peak width: 0.30 min.

Name of the compound	*B.p. °C*	*Relative retention ratio*
n-Pentane	36.2	0.378
Dichlorodifluoromethane (Freon 12)	−28.0	0.387
Dichlorotetrafluoroethane (Freon 114)	3.8	0.387
2,2-Dimethylbutane	49.7	0.387
Propane	−42.17	0.387
Propylene	−47.0	0.387
2-Bromo-2-methylpropane	73.3	0.420
2,3-Dimethylbutane	58.1	0.420
Isopentane	28.0	0.420
2-Methylbutene-2	38.4	0.420
3-Methylpentane	64.0	0.450
4-Methylpentene-2	58.0	0.450
Pentene-2	36.4	0.450
Cyclopentane	49.5	0.485
2,4-Dimethylpentane	80.5	0.485
3-Methylbutene-1	25.0	0.485
tert.-Butyl methyl ether	—	0.515
1-Chloropropene	35.0	0.515
Dimethoxymethane	44.0	0.515
Ethyl ether	34.6	0.515
n-Hexane	69.0	0.515
Hexene-1	63.49	0.515
2-Methylpentane	60.0	0.515
2-Methylpentene-1	61.5	0.515

(*Continued on p. 231*)

TABLE IV (*continued*)

Name of the compound	*B.p.* °C	*Relative retention ratio*
2-Chloropropane	35.4	0.517
3-Bromopropene	71.3	0.548
Trichlorofluoromethane (Freon 11)	24.1	0.548
Isopropyl ether	67.5	0.550
Bromoethane	38.0	0.580
Ethyl acetate	77.15	0.580
Hexene-2	67.9	0.580
Hexenes (mixed, 2 and 3)	64–67	0.580
Methyl alcohol	64.65	0.580
Methylcyclopentane	71.80	0.580
2,2,4-Trimethylpentane	99.3	0.580
2,3-Dimethylpentane	89.7	0.610
3-Methylhexane	91.85	0.610
tert.-Butyl ethyl ether	68.9	0.615
Ethyl formate	54.3	0.64
Carbon disulfide	46.3	0.645
Cyclohexane	81.4	0.645
1,1-Dimethoxyethane	64.5	0.645
Iodomethane	42.5	0.645
Isopropenylbromide	48.4	0.645
Methyl acetate	57.1	0.645
Propyl isopropyl ether	82–83	0.645
1-Chloropropane	47.2	0.678
n-Heptane	98.4	0.680
2-Bromopropane	59.6	0.710
tert.-Butyl nitrite	63.0	0.710
3-Chloropropene	44.6	0.710
Dimethylhexanes (mixed)	108–16	0.710
2-Iodopropane	89.5	0.710
Ethyl alcohol	78.5	0.740
Propionaldehyde	48.8	0.743
Dichloromethane	40.1	0.745
Heptene-2	98.0	0.750
Acetone	56.5	0.775
tert.-Butyl alcohol	82.8	0.775
1-Chloro-2-methylpropane	68.9	0.775
Ethyl isocyanate	60.0	0.775
Isobutyraldehyde	61.5	0.775
Isopropyl alcohol	82.3	0.775
1-Bromo-1-propene	60.2	0.840
Di-isobutylene	102.6	0.840
Heptene-3	95.8	0.840
Propyl ether	91.0	0.840
2,4,4-Trimethylpentene-1	101.44	0.840
2,4,4-Trimethylpentene-2	101.1	0.840
Vinyl acetate	72.3	0.840
Methylcyclohexane	100.3	0.870
2,2,5-Trimethylhexane	—	0.903
1-Bromopropane	70.9	0.935
Cyclohexene	83.0	0.935
2-Chloro-2-methylbutane	86.0	0.960
1-Chlorobutane	78.0	0.967
Methyl ethyl ketone	79.6	1.00
Isopropyl acetate	89.0	1.00
n-Octane	125.8	1.03
Butyraldehyde	75.7	1.06
1,2-Dichloroethylene (*cis*)	60.1	1.09

(Continued on p. 232)

TABLE IV (*continued*)

Name of the compound	*B.p. °C*	*Relative retention ratio*
Trichloromethane	61.26	1.10
Diethoxymethane	89.0	1.11
Acrylonitrile	78–79	1.13
Benzene	80.1	1.16
Dimethyl diketone	88.0	1.19
Propyl alcohol	97.19	1.193
1-Bromo-2-methylpropane	91.5	1.22
Octene-1	121.3	1.22
2-Bromobutane	91.3	1.225
sec.-Butyl alcohol	99.5–100	1.25
4-Methylcyclohexene	102–103	1.255
1-Chloro-3-methylbutane	98.9	1.29
Octene-2	121–22	1.32
tert.-Amyl alcohol	101.8	1.35
Ethyl nitrate	88.7	1.35
Allyl alcohol	96–97	1.355
2-Fluoroethanol	103.35	1.42
Methyl isopropyl ketone	93.0	1.42
Trichloroethylene	87.0	1.42
n-Nonane	150.79	1.48
1-Bromobutane	101.6	1.485
1,2-Dichloroethane	83.5	1.485
Thiophene	84.12	1.485
Propane nitrile	97.1	1.515
Cycloheptene	115.0	1.55
n-Propyl acetate	101.6	1.55
1-Chloropentane	108.2	1.58
Nitromethane	101.0	1.58
Ethyl acrylate	99.8	1.59
Allyl acetate	103–104	1.61
sec.-Butyl acetate	112–113	1.61
Diethyl ketone	102.7	1.61
1-Iodopropane	102.4	1.61
Butyl formate	106.8	1.67
Isopropyl nitrate	102.0	1.67
Methyl methacrylate	100.0	1.67
3-Bromopropyne	88–90	1.71
1,2-Dichloropropane	96.8	1.74
Isobutyronitrile	107–108	1.77
3-Iodopropene	103.1	1.80
Butyl alcohol	117.7	2.00
1,4-Dioxane	101.5	2.03
Crotonaldehyde	104–105	2.06
Isobutyl methyl ketone	119.0	2.06
Butyl ether	142.0	2.10
Trichloroacetaldehyde	98.0	2.13
4-Vinylcyclohexene-1	130.6	2.13
2,3-Dichloropropene	94.0	2.14
Tetrachloroethylene	121.02	2.21
2-Iodobutane	117.5	2.22
Bromotrichloromethane	104.07	2.32
Butyl acetate	126.50	2.35
1-Bromo-2-chloroethane	107.0	2.42
Ethyl carbonate	125.8	2.45
Butane nitrile	118.0	2.45
Chloropicrin	112.0	2.52
Di-isopropyl ketone	123.7	2.54

(*Continued on p. 233*)

TABLE IV (*continued*)

Name of the compound	*B.p. °C*	*Relative retention ratio*
Epichlorohydrin	117.0	2.58
2-Propyn-1-ol	114–115	2.58
Nitroethane	114.8	2.605
Propyl propionate	123.4	2.71
3-Butane nitrile	116–119	2.77
Paraldehyde	124.4	2.80
Isoamyl alcohol	130.5	2.97
n-Decane	174.0	3.00
2-Nitropropane	120.0	3.03
2-Bromo-1-chloropropane	117.0	3.06
1,1,2-Trichloroethane	113.5	3.45
Ethyl benzene	136.15	3.48
Isoamyl acetate	142.5	3.48
p-Xylene	138.35	3.74
n-Amyl alcohol	138.0	3.80
Pyridine	115.3	4.05
o-Xylene	144.41	4.35
Amyl acetate	148.0	4.40
2,4-Pentanedione	139.0	4.40
m-Xylene	139.1	4.40
Cumene	152.39	4.90
Butyl nitrite	75.0	6.70
Bromocyclohexane	163–165	7.75
Cyclohexanone	156.7	9.15
1,2,3-Trichloropropane	156.0	10.25

The relative retention times have varied between 0 % and 40 % for different compounds over the temperature differential of 20°. There is no definite pattern for this deviation. Also, in the majority of cases, the relative retention time was lower at a higher temperature. This deviation is quite obvious from the relation between the retention time and the column temperature[3].

$$\ln R_t = \text{const} + \frac{\alpha T_b}{R} \cdot \frac{1}{T} \qquad (1)$$

where R_t = the retention time,
α = a constant characteristic of the solute and the substrate,
T_b = the boiling point,
R = the gas constant,
T = the column temperature.

Each solute and the standard will give rise to one such equation. A plot of the log retention times of a solute at different temperatures against the inverse temperature will be a straight line whose slope will be given by $\alpha\, T_b/R$. Unless the slopes of the ln R_t *vs.* $1/T$ plots for different compounds are the same, *i.e.*, $\alpha\, T_b/R$ is a constant irrespective of the solute, the ratio of the retention times of different compounds at different temperatures will not be the same. It is realized that log R_t *vs.* $1/T$ or log V_g *vs.* $1/T$ plots for each compound on each column would have been most ideal identification plots. LITTLEWOOD, PHILLIPS AND PRICE[4] have shown that straight lines are obtained for alcohols, esters and aromatics by plotting log V_g against $1/T$;

V_g was named as corrected retention volume per gram by these authors[4] and is expressed as[5]:

$$V_g = \frac{273\ K}{Tc \cdot Pc} = \frac{273\ V^\circ{}_m}{T_m \cdot w} = \frac{F_m . f . d}{V_c}\ \frac{273}{T_m \cdot w} \tag{2}$$

where,:

$K = \dfrac{\text{weight of solute per ml of substrate}}{\text{weight of solute per ml of gas}}$

$V^\circ{}_m$ = the retention volume of the solute measured from air peak at the flow meter temperature T_m

T_m = temperature at the flow meter

w = weight of the substrate in the column

F_m = volumetric flow rate at temperature T_m

f = pressure gradient correction[5]

d = distance on the recorder chart between air peak and the solute peak

V_c = recorder chart speed

Because V_g is directly proportional to R_t, log R_t *vs.* $1/T$ plots will also be straight lines as is indicated by eqn. (1). LITTLEWOOD *et al.*[4] also observed that the slopes of log V_g *vs.* $1/T$ plots of the members of homologous series were the same, but the slopes of such curves due to the solutes belonging to different homologous series may not be the same. There are four possible cases: (1) log R_t *vs.* $1/T$ plot of the solute is parallel to that due to the standard solute, (2) log R_t *vs.* $1/T$ plots diverge with increasing temperature in the range studied, (3) log R_t *vs.* $1/T$ converge with increasing temperature in the range studied, and (4) these plots intersect. In the first case, R_t/R_{ts} = constant at all temperatures (R_{ts} = retention time of the standard). In the second and third cases, this ratio increases and decreases respectively. In the fourth case, the ratio first decreases, becomes one and then increases. Because these plots are straight lines, these deviations will be proportional to the change of temperature. So knowing the value of the R_t/R_{ts} ratio at two different temperatures on the same column, it should be possible to approximately calculate the R_t/R_{ts} ratio at intermediate column temperatures. In this connection, the statement by HIVELY[6] that "under very different conditions, the variation was only 5%" is somewhat misleading. The variation of retention time with the temperature is theoretically expected and is experimentally observed[5].

Such observations are consistent with those made in the present study of nearly two hundred compounds. However, as has been pointed out earlier, knowing the R_t/R_{ts} ratio at two temperatures, it should be possible to calculate the ratio at an intermediate temperature. The present data should be useful for those using the indicated columns operating in the temperature range shown in the tables.

ACKNOWLEDGEMENT

This investigation was supported by Research Grant S–43, Study Section of Sanitary Engineering and Occupational Health, Division of Research Grants, Public Health Service.

SUMMARY

The relative retention times of organic compounds on tri-*m*-tolyl phosphate and β,β'-oxydipropionitrile columns have been determined and tabulated. The method for the use of these tables has been suggested. Through the use of the two columns with subsequent reference to the data on the respective retention times, a wide variety of compounds can be tentatively identified.

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THE STRUCTURAL IDENTIFICATION OF SOME NATURALLY OCCURRING BRANCHED CHAIN FATTY ALDEHYDES

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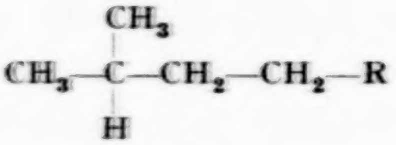
(Received December 23rd, 1960)

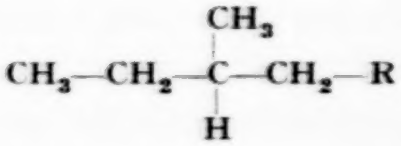
A systematic study of the distribution patterns of the fatty acids and fatty aldehydes of the lecithin- and kephalin-phospholipid fractions isolated from selected animal tissues[1] has revealed the complex nature of some of the mixtures of aldehydes. The aldehydes were separated and identified by gas–liquid chromatography[2]. A large proportion of the aldehyde fraction isolated from ox-spleen and from ox-liver was composed of compounds which, on the basis of their chromatographic behaviour[2], were identified as branched chain aldehydes.

Branched chain acids were first shown to be present in animal fats by **SHORLAND**[3] and co-workers and were identified as belonging to the iso-(I) and (+)-anteiso-(II) series of saturated branched chain compounds.



$$\mathrm{CH_3{-}\underset{H}{\overset{CH_3}{C}}{-}CH_2{-}CH_2{-}R}$$

(I)



$$\mathrm{CH_3{-}CH_2{-}\underset{H}{\overset{CH_3}{C}}{-}CH_2{-}R}$$

(II)

$$R = (CH_2)_n COOH$$

It is reasonable to suppose that the branched chain aldehydes would also have the same iso- or anteiso-structure since the biosynthesis of both fatty acids and fatty aldehydes probably proceeds initially from the same set of carbon "fragments".

The gas chromatographic behaviour of methyl esters of authentic members of the iso- and anteiso-series of fatty acids has been studied and reliable retention data on different stationary phases have been published[4,5]. The branched chain aldehydes were therefore oxidized to the corresponding acids and the chromatographic behaviour of the methyl esters of these acids compared with that of authentic iso- and anteiso-markers. The results showed that the behaviour of the methyl esters of the acids derived from the aldehydes was identical to that of the authentic iso- and anteiso-markers on both polar and non-polar stationary phases. On the basis of the evidence the naturally occurring branched chain aldehydes belong therefore to the iso- and anteiso-series.

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EXPERIMENTAL

Materials

The methyl esters of 12-methyltridecanoic acid, 12-methyltetradecanoic acid, 14-methylpentadecanoic acid, 16-methylheptadecanoic acid and 16-methyloctadecanoic acid were prepared from authentic samples kindly donated by Dr. A. H. MILBURN.

The aldehydes were isolated from the choline plasmalogen fraction of ox-spleen and converted to their dimethyl acetal derivatives by methods described previously[1,2].

The quantitative oxidation of the aldehydes to the corresponding acids was carried out by adapting the excellent method of POLLARD, CHIBNALL AND PIPER[6] for the oxidation of long chain alcohols to acids. The aldehyde dimethyl acetals (0.1 ml) were dissolved in 4.0 ml 90% acetic acid, 0.25 ml 5 N anhydrous methanolic HCl was added and the solution was heated for 1 h at 90–100°. The solution was diluted with one volume of water and extracted with (4 ×) 1.5 ml light petroleum

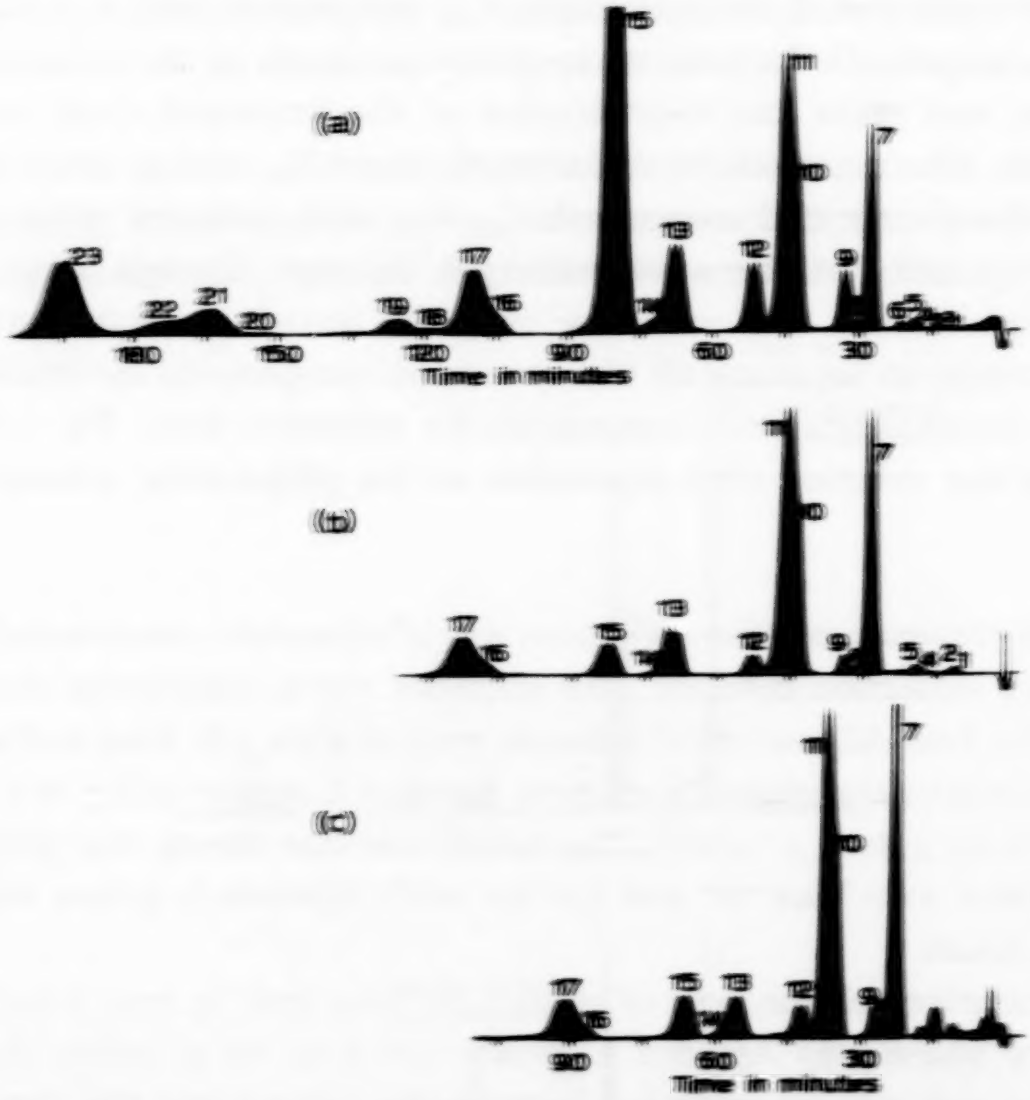

Fig. 1. 4 ft. column with Apiezon L grease as stationary phase at 190°. Argon pressure, column inlet, 103 cm Hg; outlet pressure, atmospheric. (a) Analysis of dimethyl acetals of aldehydes isolated from ox-spleen choline plasmalogen. (b) Analysis of dimethyl acetals of aldehydes isolated from ox-spleen choline plasmalogen after removing most of the straight chain saturated and unsaturated components. (c) Analysis of the same mixture as in (b) after the aldehyde dimethyl acetals had been converted by oxidation to the corresponding acid methyl esters. Peaks: (1) and (2) branched dodecanoic; (3) *n*-dodecanoic; (4) and (5) branched tridecanoic; (6) *n*-tridecanoic; (7) and (8) branched tetradecanoic; (9) *n*-tetradecanoic; (10) and (11) branched pentadecanoic; (12) *n*-pentadecanoic; (13) branched hexadecanoic; (14) branched hexadecanoic + monounsaturated hexadecanoic; (15) *n*-hexadecanoic; (16) and (17) branched heptadecanoic; (18) unknown; (19) *n*-heptadecanoic; (20) linoleic; (21) oleic; (22) isooleic; (23) *n*-octadecanoic.

(b.p. 40–60°). The light petroleum was removed under vacuum and the residue was dissolved in 2.5 ml glacial acetic acid. The solution was warmed slightly (approx. 40°) and small amounts of chromium trioxide in glacial acetic acid were added until excess was present (solution remained red-brown instead of gradually changing to green). The solution was then poured into water (2 volumes) and extracted with benzene. The benzene solution was washed with water, evaporated to dryness and the residue was dissolved in dry ether. Sodium methoxide was gradually added and a precipitate of the sodium salts of the fatty acids was formed. This was centrifuged, washed with a little light petroleum and dissolved in water. The acids were liberated with HCl, extracted with ether, dried over anhydrous sodium sulphate and finally converted to their methyl esters with anhydrous methanolic HCl.

Gas chromatographic analysis of dimethyl acetals of the aldehydes isolated from ox-spleen choline plasmalogen (Fig. 1(a)) indicated that the branched chain aldehydes were in the range C_{13}–C_{17} though there were considerable amounts of saturated and unsaturated straight chain C_{18} compounds and a small amount of saturated C_{20} compound[7]. In order to keep side products of the oxidation procedure to a minimum and make the identification of the branched chain compounds as easy as possible, all compounds of chain length above C_{17} and as much as possible of the straight chain saturated compounds C_{12}–C_{17} were removed prior to oxidation by passing a quantity of the whole aldehyde fraction through a preparative gas chromatographic column and collecting only the branched chain components. No attempt was made to separate all the saturated components as small amounts of these provide internal reference compounds for retention data. Fig. 1(b) shows the composition of the mixture after separation on the preparative column.

Columns

The gas–liquid chromatographic apparatus was a laboratory constructed model using the argon β-ray ionization detector. The amplifier was a commercial one supplied by W. G. Pye & Co. Ltd. All analytical columns were of glass 4 ft. long and 4 mm internal diameter. The stationary phases used were Apiezon L grease (1.8 g to 7 g celite) and Reoplex 400 (1.05 g to 7 g celite). The celite (100–120 mesh) was prepared by the method of JAMES AND MARTIN[8] and for use with Apiezon L grease was pretreated with alcoholic alkali.

The preparative column was of glass 5 ft. long and 13 mm internal diameter. The stationary phase was Apiezon L grease (18 g to 60 g celite) (80–100 mesh). A small bleeder unit was incorporated between the column and the argon detector so that only a controlled percentage of the gas outflow from the column, further diluted with pure argon, actually passed through the detector. Thus whatever the size of the sample loaded on the column no overloading of the detector occurred.

RESULTS

The composition of the mixture of aldehyde dimethyl acetals before oxidation of the aldehydes to the corresponding acids is shown in Fig. 1(b). The interpretation of whether the recording of the chromatographic separation of the aldehyde mixture was showing the presence of small amounts of components (labelled 8 and 14) or excessive tailing of components 7 and 13 was checked by chromatographing the mixture on Apiezon L at 160°. The increase in the separation efficiency of the column at the lower temperature was sufficient to show that components 7 and 13 were not tailing and that components 8 and 14 were in fact real.

The composition of the mixture of methyl esters of the acids obtained by oxidation of the aldehydes is shown in Fig. 1(c) and a comparison with Fig. 1(b) shows the marked difference in retention volumes of acetals and esters of the same carbon number. The appearance of a number of small fast-running components after oxidation was probably the result of breakdown across the double bond of some palmitoleic aldehyde present in the mixture. A decrease in the height of the peak representing components 13 and 14 (Fig. 1(c)) on Apiezon L and palmitoleic aldehyde on Reoplex 400 after oxidation as compared with the peak heights of other components indicated that the unsaturated components do suffer some breakdown during the oxidation procedure.

A number of samples of the mixture of acid methyl esters, each plus a different authentic branched chain marker, were chromatographed on Apiezon L (Fig. 2) and Reoplex 400 stationary phases. The carbon numbers of the acid esters were

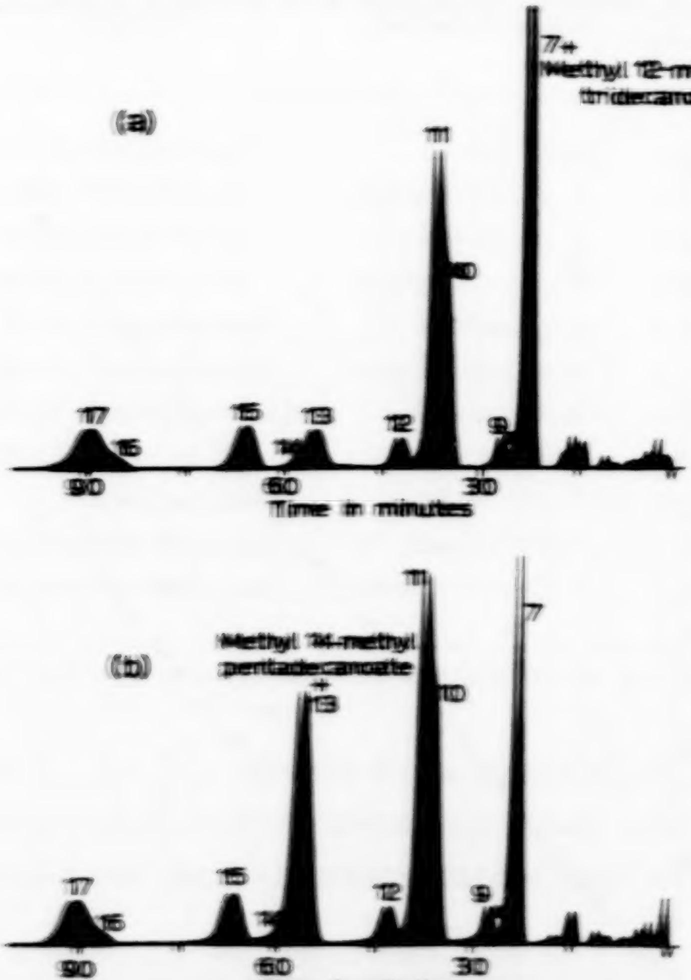

Fig. 2. Analysis of the methyl esters of acids (Fig. 1(c)) obtained by oxidizing a mixture of aldehydes (Fig. 1(b)) isolated from ox-spleen choline plasmalogen. Column conditions as for Fig. 1. (a) Mixture plus methyl 12-methyltridecanoate. (b) Mixture plus methyl 14-methylpentadecanoate. Peak identification as for Fig. 1.

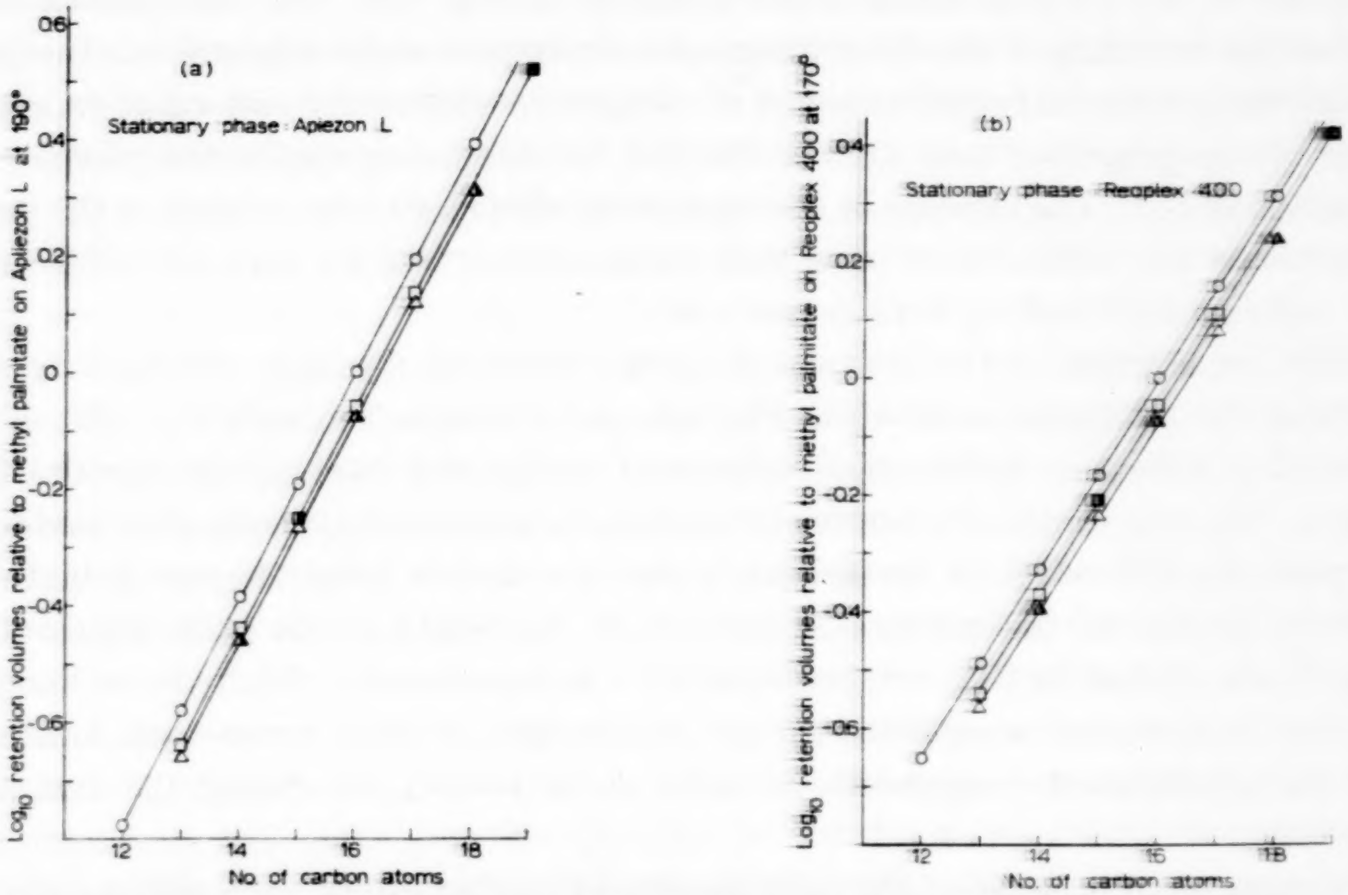

Fig. 3. Retention volumes of some saturated straight chain and branched chain fatty acids relative to methyl palmitate. Straight chain saturated acids ○ ; pure synthetic branched chain acids of the iso-series ▲ ; pure synthetic branched chain acids of the anteiso-series ■ ; branched chain acids obtained by oxidation of branched chain aldehydes isolated from ox-spleen choline plasmalogen △, □ ; synthetic and isolated compounds with identical retention volumes ▲, ■.

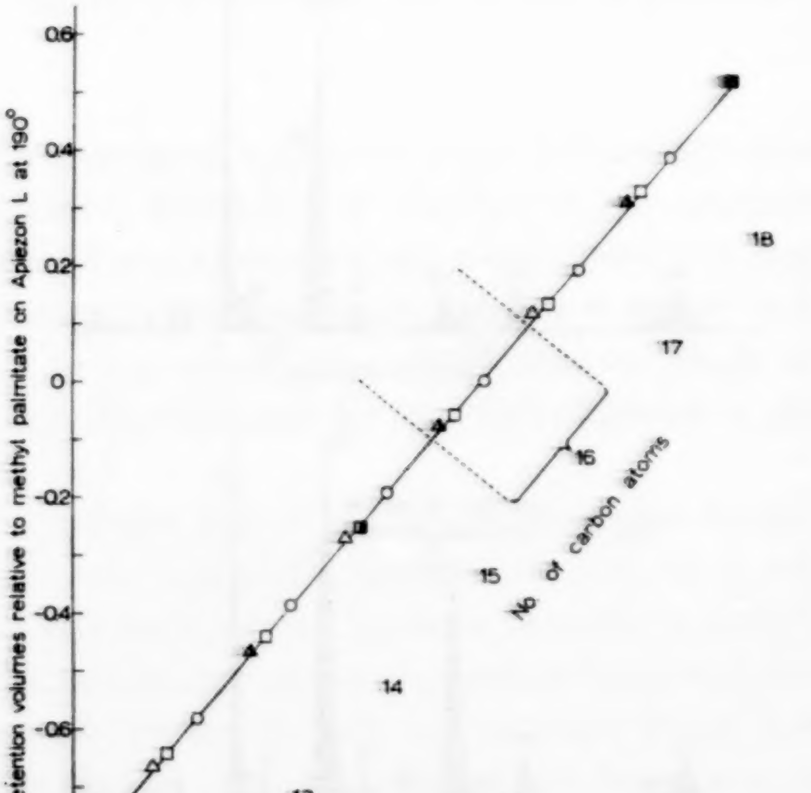

Fig. 4. Log$_{10}$ relative retention volumes of saturated straight and branched chain methyl esters on Apiezon L plotted against log$_{10}$ relative retention volumes on Reoplex 400. Symbols as for Fig. 3.

plotted against the logarithms of their retention volumes relative to methyl palmitate (Fig. 3).

The closed symbols represent the plots of synthetic branched chain acids of known structure and the half-closed symbols represent plots of the synthetic acid and of a component of the mixture with an identical retention volume. From a comparison of their chromatographic behaviour with that of the pure synthetic compounds it is apparent that all the branched chain acids derived from the aldehydes belong to either the iso- or anteiso-series of compounds. Additional confirmation of their saturated branched chain structure was obtained by plotting the logarithms of the relative retention volumes on Apiezon L stationary phase against those on Reoplex 400 stationary phase (Fig. 4).

These results provide sound evidence for stating that, like the naturally occurring branched chain acids, the naturally occurring branched chain aldehydes belong to the iso- and anteiso-series of compounds. The relative retention volumes and structures of a number of branched chain aldehydes found in ox-spleen and ox-liver are given in Table I. It was found that the retention volumes of the aldehyde dimethyl acetals over the range C_{12} to C_{17} relative to palmitaldehyde dimethyl acetal were identical to the retention volumes of their corresponding acid methyl esters relative to methyl palmitate under the same chromatographic conditions.

TABLE I

RETENTION VOLUMES* OF SOME BRANCHED CHAIN ALDEHYDE DIMETHYL ACETALS RELATIVE TO PALMITALDEHYDE DIMETHYL ACETAL

Peak No. Fig. 1(a)	*Aldehyde*	*Shorthand designation*	*Stationary phase*	
			Apiezon L at 190°	*Reoplex 400 at 170°*
4	11-Methyldodecanal	iso-br. 13:0	0.218	0.270
5	10-Methyldodecanal	anteiso-br. 13:0	0.228	0.286
7	12-Methyltridecanal	iso-br. 14:0	0.348	0.400
8	11-Methyltridecanal	anteiso-br. 14:0	0.362	0.423
10	13-Methyltetradecanal	iso-br. 15:0	0.535	0.580
11	12-Methyltetradecanal	anteiso-br. 15:0	0.560	0.616
13	14-Methylpentadecanal	iso-br. 16:0	0.840	0.840
14	13-Methylpentadecanal	anteiso-br. 16:0	0.870	0.892
15	Hexadecanal	16:0	1.00	1.00
16	15-Methylhexadecanal	iso-br. 17:0	1.31	1.20
17	14-Methylhexadecanal	anteiso-br. 17:0	1.36	1.278

* All retention volumes were measured from the middle of the air peak.

ACKNOWLEDGEMENT

I should like to thank the British Empire Cancer Campaign for their support of this work.

SUMMARY

Branched chain aldehydes isolated from animal tissues have been shown to belong to either the iso- or anteiso-series of compounds by oxidising them to the corresponding acids and comparing the gas chromatographic behaviour of these acids with that of pure synthetic acids of known structure.

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INCREASED EFFICIENCY FOR LARGE-VOLUME GAS CHROMATOGRAPHIC SAMPLES

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(Received December 21st, 1960)

INTRODUCTION

The effect of sample-size broadening has long been recognized in gas chromatography. Sample-size broadening should not be confused with peak diffusion due to large retention volumes resulting from long lengths of columns or large numbers of theoretical plates. Fig. 1 illustrates the progressive broadening and flattening of a peak as the sample size is increased. Ideally, the sample should be injected as a small slug, so as to occupy initially a space equivalent to only a small percentage of the plates in the column. The number of plates which may be nonrestrictively taken up by the sample increases as the number of plates in the column increases[1].

In regard to peak widths and shapes, DAL NOGARE[2] pointed out that determination of true column efficiency should involve the measurement of band spreading due only to the column and not to extra-column factors. JOHNSON AND STROSS[3] separated the effects on peak width into column and noncolumn factors. They also theoretically calculated and plotted noncolumn peaks (peaks due to the apparatus without the column). PURNELL[4] studied the effect of sample size of isopropanol on the efficiency of a firebrick column coated with 20% polyethylene glycol. WHATNOUGH[5] determined the effect on peak shape as larger quantities of methane were injected into an absorption column. PORTER, DEAL AND STROSS[6] theoretically calculated and plotted curves for varying sample sizes in the case of plug-type charging of a solute-gas mixture. Plug injection *versus* exponential injection for large samples was studied by DE WET AND PRETORIUS[7], who showed that higher column efficiencies may be obtained by introducing the sample in as concentrated a form as possible.

The purpose of this paper is limited to the effect of one factor on efficiency, *viz.*, the effect of an enlarged section in a packed column on peak shape, especially in those instances involving large samples of gases. The effect of increasing the diameter, in the case of uniform-diameter columns, has been studied by DE WET AND PRETORIUS[8]. Very little has been reported on the effects of cross-sectional geometry on column efficiency, and it appears that the use of enlarged sections may afford excellent opportunities for easily improving efficiencies.

EXPERIMENTAL

Columns

Two columns were compared in this study. One column was made of 1/4-in. uniform-diameter copper tubing, 12 ft. long, and packed with 30/60-mesh acid-washed Chromosorb coated with tri-*m*-cresyl phosphate (20% by weight). The second column was identical to the first, with this exception: a 4 1/2-in. length of 3/8-in. diameter copper tubing, filled with packing identical to that in the columns, was attached to the forefront of the column[9].

Conditions of operation

Helium was used as a carrier gas in these studies, at an exit flow rate of 30 ml/min. The temperature of the two columns was maintained at 40°, as was the temperature of the thermal conductivity (thermistor) cell. All samples were injected through a rubber septum with a hypodermic syringe as rapidly as possible to simulate a slug-type charge.

Retention times were measured from the point of injection to the midpoint of the peak. Peak base lengths were measured between the intercepts of the tangents to the peak at the base line. Column efficiency is expressed in terms of numbers of theoretical plates, as recommended by a special committee of the International Union of Pure and Applied Chemistry[10], in July 1959.

$$n = 16 (t_R/W)^2$$

where n = number of theoretical plates
t_R = retention time
W = peak base measured at points where the extended tangents intersect the base line.

EXPERIMENTAL DATA AND DISCUSSION

The effect of varying the sample size, for a plug-type charge, is shown in Fig. 1 for samples of ethane. A small decrease in retention time, not indicated, was noted for each increase in sample volume. The series of curves in the figure resemble those calculated theoretically by PORTER, DEAL AND STROSS for plug-type charging of a solute-gas mixture[6]. As pointed out by these authors, the flattened top on the upper curve corresponds to the case in which the sample size is so large that it fills a substantial part of the column before the charging step is complete.

Fig. 2 shows the effect of using an expanded-section column on the elution curve for 40-ml samples of ethane. All conditions of operation and column construction were identical with the exception of the addition of a 4 1/2-in. length of 3/8-in. diameter packed tube at the forefront of one column. A marked increase in efficiency was noted with the expanded-section; a similar increase was also present when the column was inverted so that the expanded section was next to the thermal conductivity detector used in this work. The total peak area is decreased, as is the retention time (the latter

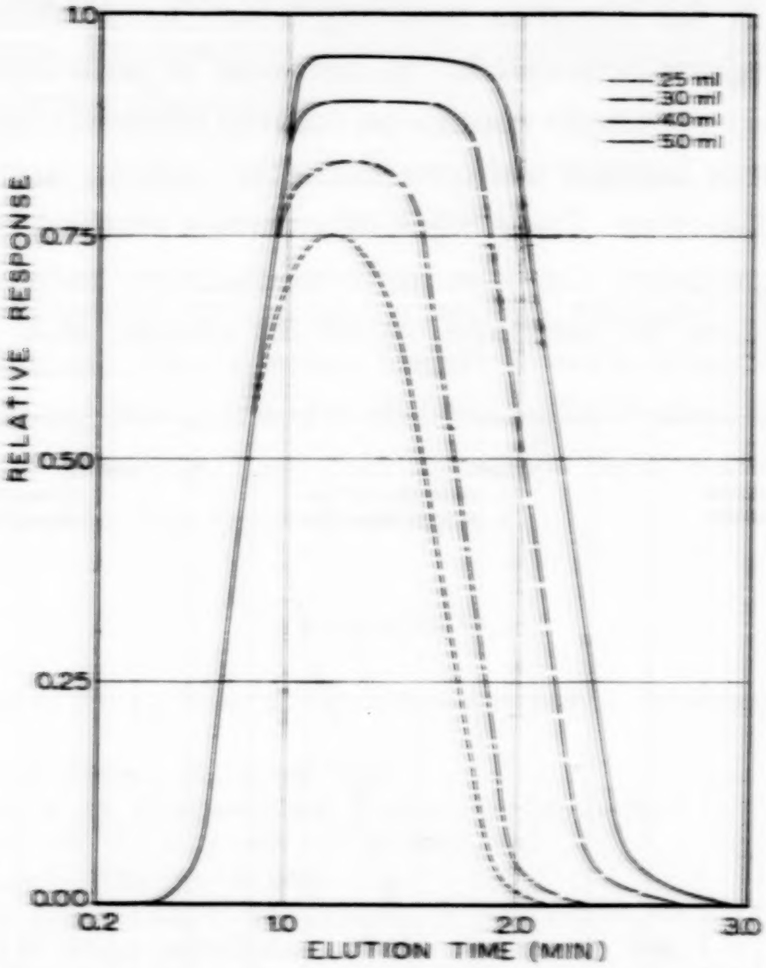

Fig. 1. Elution curves for variable samples of plug-injected ethane.

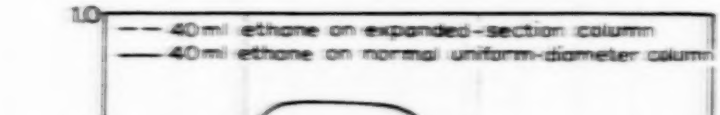

Fig. 2. Elution curves for equal-volume samples analyzed on expanded-section and normal columns. A short, packed, enlarged section was attached to the forefront of one of the columns; otherwise, both columns were identical. Conditions of operation were identical.

is not indicated in Fig. 2), for the peak resulting from the expanded-section column. The reason for the anomalous behavior in the decrease in peak area is not evident.

Fig. 3 shows the effect of sample volume on column efficiency for methane, ethane, and propane, with both a normal uniform-diameter column and a similar column containing an expanded section. The ranges of volumes studied increase in order of methane, ethane, and propane; *i.e.*, the uniform-diameter column gave maximum nonflattened peaks of 15 ml for methane, 25 ml for ethane, and 35 ml for propane.

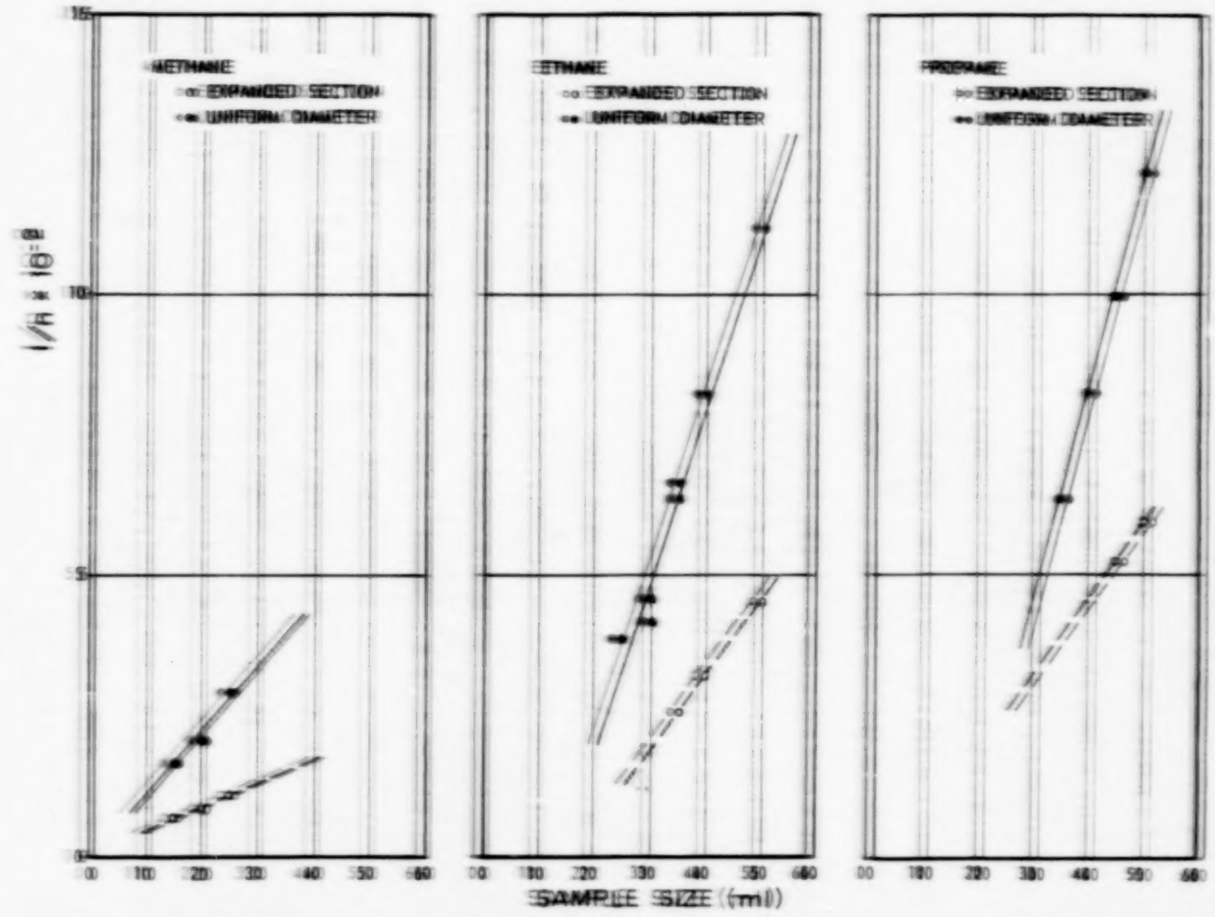

Fig. 3. Comparison of effects of sample volume on column efficiency for methane, ethane, and propane.

Also, these maximum values for usable sample sizes were increased, by the addition of the enlarged packed section, to 25, 40, and 50 ml respectively.

The effect of increasing volume on peak shape has been treated theoretically in detail[6,7,11], as has the effect of increasing diameter in uniform-diameter columns[8]. The beneficial effect of the enlarged (expanded) section in the work being reported is clearly evident. The maximum volume which could be handled was increased by a factor of about 1.5 by use of the expanded section. Since only a short section of increased-diameter tubing was used, the value of 1.5 seems reasonable compared to the value of about 2.3 calculated for a full length column on the basis that the volume of sample which can be handled by a column at a chosen efficiency is proportional to the square of the column diameter[8]. The enlarged section acts as a collector to compress the sample under a given peak into a shorter plug. This is probably due to increased lateral diffusion in the enlarged section.

The addition of expanded sections to normal uniform-diameter columns may prove beneficial in large scale preparative columns, or even perhaps capillary columns, by enhancing the efficiency and resolution. Enlarged sections have been used in this laboratory to improve the analysis of small quantities of multicomponent mixtures diluted by large volumes of carrier gas.

SUMMARY

The authors studied the effect of adding a packed, enlarged-diameter section to a gas chromatographic column. Two columns, identical in all respects except for one having an enlarged section, exhibit markedly different efficiencies. The use of the enlarged section increases the efficiency and also increases the maximum volume of sample which may be handled by the packed column.

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THE PREPARATION OF CARRIER-FREE ^{115}In BY A REVERSE PHASE PARTITION TECHNIQUE

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(Received December 6th, 1960)

INTRODUCTION

The preparation of carrier-free tracers by the separation of a daughter element from its parent, has frequently necessitated complicated chemical procedures, but in a number of cases the method has been considerably simplified by the use of ion exchange techniques, which have allowed selective elution of the tracer required. Recently it has been shown that solutions of chelating agents in organic solvents may be retained on a solid supporting medium[1], and providing that the stabilities of the complexes formed by the chelating agent with parent and daughter differ sufficiently, it should be possible to carry out separation by selective elution in a manner exactly similar to that of the ion exchange technique.

Since the most convenient type of separation is that provided by elution of the daughter, the metal to be eluted should partition in favour of the eluting solvent, giving an R_F value approaching 1, whilst the parent should be retained by the support with an R_F value of near 0. This ensures that the minimum volume of eluting agent is required to effect the separation, and that contamination of the effluent by the parent is precluded.

This type of method has been used to separate carrier-free ^{115}In from parent ^{115}Cd, a procedure previously involving either a precipitation technique[2] or solvent extraction[3], since the greater stability of the complex of cadmium with dithizone (diphenyl thiocarbazone) enables the indium to be eluted preferentially from a dithizone column which initially contains both metals.

The dithizone column was made up as previously described[1] by retaining a solution of dithizone in an organic solvent on silica gel. In order to carry out column operation under conditions which approach equilibrium as nearly as possible, the organic solvent should favour quick reaction between metal and chelating agent. Chloroform and carbon tetrachloride are the two solvents most widely used for dithizone, and whilst carbon tetrachloride solutions tend, in general, to react more quickly with metals in acid solution[4] the greater solubility of dithizone in chloroform enables a support of higher capacity to be prepared. Consequently in these investigations, experiments were carried out using both chloroform and carbon tetrachloride as organic solvent.

EXPERIMENTAL

Silica gel, 85–120 mesh, was prepared from sodium silicate by precipitation with 10 *N* hydrochloric acid and, after sieving, was purified by washing, first with successive volumes of concentrated hydrochloric acid until the acid remained colourless, and then with a strong ethylenediaminetetraacetic acid solution of pH 5. The gel was finally washed with water, alcohol and ether, and dried at 110°.[1]

A solution of purified dithizone[5] in organic solvent was added to the silica gel, and after thoroughly stirring the mixture, solvent was evaporated off with compressed air until a freely running powder was obtained. This powder was stored in a desiccator over organic solvent in the dark, and before use was always air dried to allow any surplus solvent to evaporate. The support was slurried with a *N* sodium acetate–hydrochloric acid buffer pH 5 and the column made up in the usual way.

The cadmium–indium mixture was retained as a thin band at the top of the column when the metals, in pH 5 buffer, were added as influent to the column.

After washing with pH 5 buffer the column was eluted with *N*/500 perchloric acid and the effluent from the column monitored for activity. In all cases the column size was 2.4 cm^2 × 4 cm and the flow rate was 0.15 ml/cm^2/min. The aqueous phases were presaturated with the organic solvent used on the silica gel.

The dithizone content of a given weight of support was determined by shaking the support with successive volumes of dilute aqueous ammonia until all the dithizone had been transferred into the aqueous phase, acidifying the combined aqueous extracts, and dissolving precipitated dithizone in carbon tetrachloride.

The optical density of the organic layer was measured and, using a value of $34.6 \cdot 10^3$ for the extinction coefficient of dithizone in carbon tetrachloride[6], the dithizone content of the column material calculated.

DISCUSSION

The behaviour of a metal M^{n+}, which forms a chelate in the aqueous phase of a two-phase system with a chelating agent HL according to the equation:

$$M^{n+} + nHL \rightleftharpoons ML_n + nH^+ \qquad (1)$$

has been shown to be given by[7]:

$$\frac{[ML_n]_o}{[M^{n+}]} = K \frac{P_c}{P_r{}^n} \frac{[HL]_o^n}{[H^+]^n} = K' \frac{[HL]_o^n}{[H^+]^n} \qquad (2)$$

where P_c and P_r are the partition coefficients of the chelate and chelating agent respectively, such that:

$$P_c = \frac{[ML_n]_o}{[ML_n]} \text{ and } P_r = \frac{[HL]_o}{[HL]}$$

and K is the equilibrium constant for eqn. (1). Square brackets represent concentrations, which are used in place of activities for solutions of constant ionic strength, and the subscript o denotes species present in the organic phase. It is assumed that no intermediate chelate species are formed and that other complexes are absent. Under these circumstances $[\mathrm{ML}_n]_o/[\mathrm{M}^{n+}]$ may be identified with the fraction of metal extracted.

In the case of non-extractable complexes being formed in the aqueous phase between the metal and another anion, the fraction extracted E, where: $E = \Sigma[\mathrm{M}]_o/\Sigma[\mathrm{M}]$ is now given by

$$E = K'' \frac{[\mathrm{HL}]_o^n}{[\mathrm{H}^+]^n} \qquad (2a)$$

but K'' now refers to a given anion concentration in the aqueous phase.

If, during the chromatographic elution of a metal, partitioning between a stationary phase and a mobile liquid, d_m and d_s are the distances travelled by metal and solvent front respectively, d_m will be proportional to the solubility of the metal in the mobile solvent whilst $d_s - d_m$ will be proportional to the solubility of the metal in the stationary phase[8].

Thus:

$$\frac{d_m}{d_s - d_m} = \frac{k}{E} \qquad (3)$$

where k is a constant accounting for the ratio of the cross-section of the moving and stationary phases.

Now

$$R_F = \frac{d_m}{d_s} \qquad (4)$$

so by combining eqns. (2a), (3) and (4):

$$\frac{1}{R_F} = 1 + \frac{K''}{k} \frac{[\mathrm{HL}]_o^n}{[\mathrm{H}^+]^n} \qquad (5)$$

provided eqn. (2a) represents the behaviour of a chelating agent retained on a solid support material.

From eqn. (5) it follows that an increase in R_F would result from either an increase in the hydrogen ion concentration, or a decrease in the ligand acid concentration. A high value of $[\mathrm{HL}]_o$ will be required to provide a column with as large a capacity as possible, consequently the R_F value may be most conveniently varied either by alteration of the hydrogen ion concentration or by the use of water-soluble complexing agents which form non-extractable competitive complexes with the metal, and result in the lowering of the value of K''. For the separation of two metals, conditions must be chosen so that one metal can be eluted without contamination by the other, and it was found that indium could be selectively removed from a cadmium–indium mixture, retained on a dithizone column, with $N/500$ perchloric acid. Fig. 1 gives a plot of the activity of the effluent against effluent volume for the cases where carbon tetrachloride (curve A) and chloroform (curve B) were used as organic solvents. γ-Ray spectrometry and half-life determinations showed that the activity was due solely to 4.5 h ^{115}In and no trace of cadmium could be detected.

The peak effluent volume is smaller when carbon tetrachloride is used on the silica gel, but curve B more nearly approaches the "bell-shaped curves" expected for Gaussian distribution. The higher value of $[HL]_o$ attained with chloroform as solvent ($6.08 \cdot 10^{-3}$ mols dithizone per kg of support as opposed to $1.6 \cdot 10^{-3}$ mols/kg) would be expected to favour a linear extraction isotherm and also to offset any increase in the value of K' that might occur if the chloroform was replaced by carbon tetrachloride. The dependence of the extraction process upon the presence of dithizone was verified by passing the cadmium-indium mixture in pH 5 buffer down a column of silica gel which had been treated with organic solvent but no dithizone. Under these conditions the metals were not retained by the column but passed through with the mobile phase and appeared in the effluent as soon as the liquid retained in the column had been displaced.

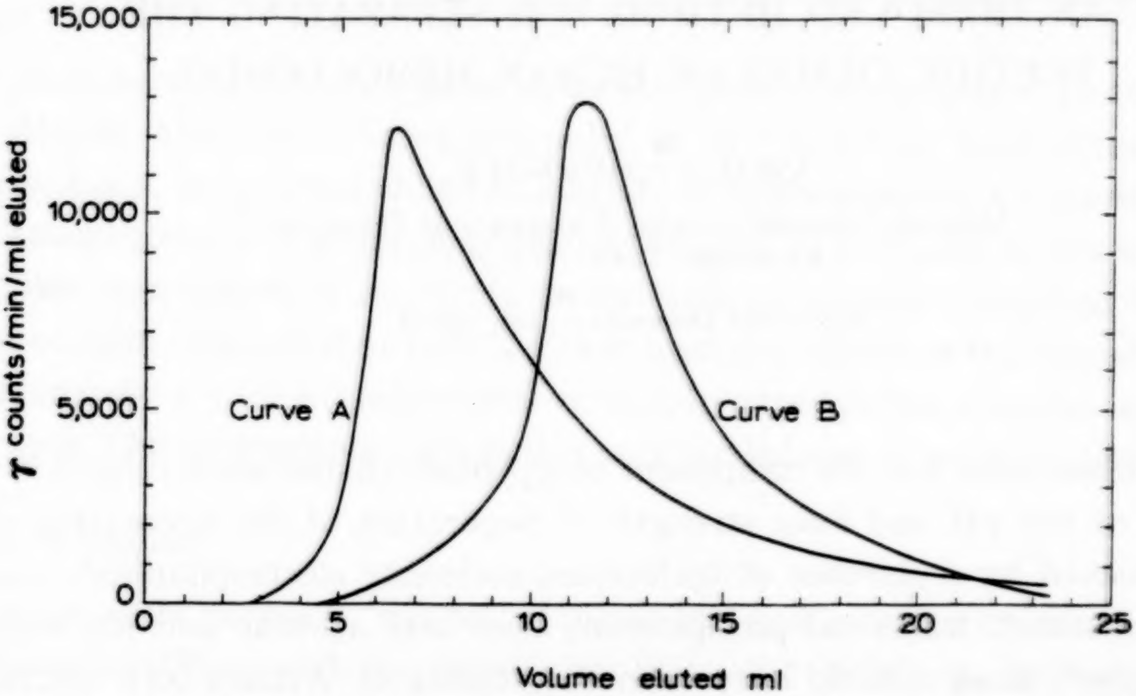

Fig. 1. Plot of activity against effluent volume for the elution of indium with perchloric acid. For curve A the organic solvent was carbon tetrachloride, for curve B, chloroform.

SUMMARY

A column of a solution of a chelating agent (dithizone) retained on silica gel, has been used to separate ^{115}In from the parent cadmium. The higher stability of the complex of cadmium with dithizone enables the indium to be preferentially eluted with dilute perchloric acid, whilst the cadmium, which remains complexed under these conditions, stays on the column.

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THE AMINO ACID COMPOSITION OF HEMOGLOBIN

I. AN IMPROVED METHOD FOR SEPARATING THE PEPTIDE CHAINS OF HUMAN HEMOGLOBIN*

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(Received December 21st, 1960)

Hemoglobin dissociates into its component polypeptide chains when subjected to an environment of low pH and ionic strength[1-3]. Separation of the dissociated chains has been achieved by a number of techniques including electrophoresis[4], countercurrent distribution[5], fractional precipitation from acid acetone and ion exchange chromatography[6]. Most notable have been the studies of WILSON AND SMITH who separated the α and β chains of horse globin by elution from Amberlite IRC-50 resin with the use of a urea solution of increasing molarity at low pH[6]. Subsequently, similar techniques have been used to separate and isolate the α, β, and γ (and possibly δ) chains occurring in human hemoglobin[7,8] and N-terminal group analyses have permitted their more precise characterization. Although the technique of WILSON AND SMITH has proven highly satisfactory for clear cut separation of the α and β chains of horse globin, much less distinct separations were obtained when this method was applied to human globin. As a consequence of the overlap of the eluted chains and the resulting cross contamination, a significant amount of material must be sacrificed to ensure a modest degree of purity of the fractions, often a serious problem when dealing with the small amounts of protein available with some of the rare types of hemoglobin. A modification of the urea gradient method of WILSON AND SMITH, which results in almost complete separation of the two types of chains of human hemoglobin and their isolation in relatively pure form in high yield, is described in this communication. The chief modification involves elution by an "interrupted" or "intermittent" urea gradient in place of the continuously variable gradient of WILSON AND SMITH.

METHODS

Hemoglobin was prepared from washed, lysed erythrocytes and purified by one of several methods, including precipitation or crystallization from ammonium sulfate solutions, elution from starch after starch block electrophoresis[9] or by denaturation with highly alkaline solutions (for Hgb F isolation)[10]. Globin was prepared from either the crystallized or purified material by the acid-acetone method of Anson and Mirsky[11]. To a 5 % solution of the hemoglobin, 10 to 15 vol. of acetone (containing 2.5 g of oxalic acid per 100 ml of acetone) were added slowly with constant stirring. Following filtration under gentle suction, the globin cake was washed repeatedly with acetone until free of heme and oxalate, air dried, and ground into a finely divided powder.

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* These studies were carried out with the support of USPHS grant No. A2956 from the National Institutes of Health, Bethesda, Md.

Amberlite resin, CG 50 type 2, in its acid phase, was prepared by the method of Hirs, Moore and Stein[12] and suspended in 11.7 % formic acid. Chromatographic columns, 1.9 × 90 cm, were filled to a height of approximately 60 cm with the resin. From 500 to 700 mg of globin may be fractionated on a column of these dimensions. The globin was dissolved in 11.7 % formic acid (10 mg/ml), adsorbed with a fresh aliquot of resin suspended in 11.7 % formic acid and added to the top of the column. Approximately 1.5 ml of sedimented resin per ml of globin solution were used for adsorption. Chromatographic separation was carried out at room temperature.

Elution techniques

For comparative purposes, the technique of Wilson and Smith was followed with only minor modifications[6]. Approximately 1600 ml of 2 *M* urea, adjusted to pH 1.9 with concentrated HCl, were placed into a two liter bottle and 3000 ml of 8 *M* urea, pH 1.9, into a four liter bottle. Six hundred ml of the 2 *M* urea were passed through the column at the rate of approximately 2 ml/min and the effluent collected in 20 or 25 ml aliquots. Gradient elution was then started by the dropwise addition with stirring of the 8 *M* urea solution to the 1000 ml of solution remaining in the smaller bottle. After 2000 to 2500 ml of solution had passed through the column, the gradient was stopped and 8 *M* urea was utilized for the final phase of elution. The optical density of each tube was read at 280 mμ and the results graphed as in Fig. 1.

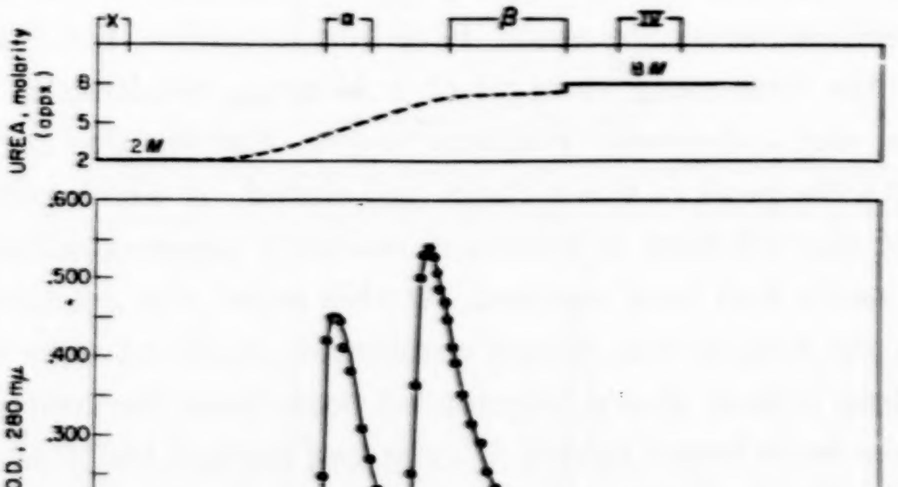

Fig. 1. Separation of the α and β chains of human globin by the method of Wilson and Smith[6]. 650 mg globin A; 20 ml fractions; pH 1.9; IRC-50 column; continuous urea gradient.

The modified technique employed in this study differs from that of **Wilson and Smith** in several respects. In general, solutions of 2 *M* and 8 *M* urea were prepared as above. 2000 ml of the 2 *M* urea and 3000 ml of the 8 *M* urea were introduced into appropriately sized bottles. The effluent from the column was continuously monitored by a direct recording U.V. detector (280 mμ)* (Fig. 2). For convenience, elution was started at the end of the working day and 1000 ml of the 2 *M* urea was permitted to wash through the column slowly overnight (approx. 1 ml/min). Similar results, however, were obtained by passing through the column only enough of the 2 *M* urea solution to elute the initial fractions which appear at the beginning of each run (usually 300 to 400 ml) at which time the absorption curve of the effluent will have returned nearly to its base line value. Appropriate adjustment of the starting volume of the 2 *M* urea should, however, be made. Five hundred ml of the 8 *M* urea solution were then added to the remaining 1000 ml of 2 *M* urea, resulting in a solution of 4 molar concentration, and a dropwise gradient begun. Elution was carried out at the rate of 2 ml/min until the peak of the α chain had eluted, at which point the gradient was interrupted and the solution of constant molarity (approximately 5.3 *M* urea) continued until the nadir had been reached. At this point, the gradient was resumed and continued until the β chain was almost completely removed after which 8 *M* urea was permitted to elute a final, sharp, pigmented peak from the column.

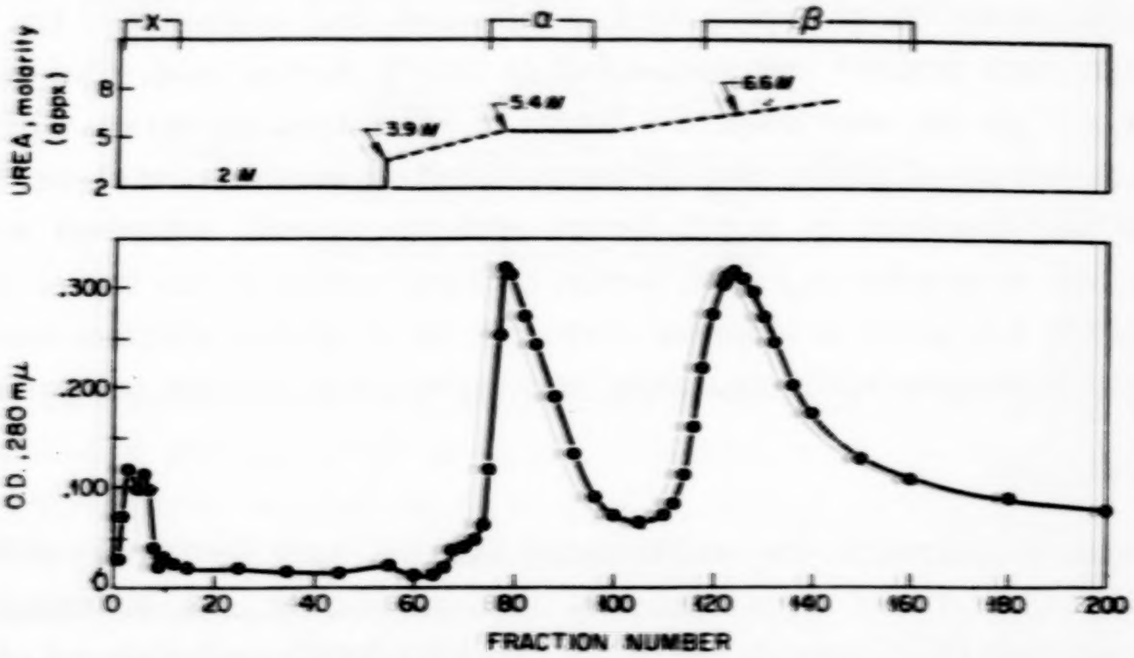

Fig. 2. Separation of the α and β chains of globin A by the interrupted gradient technique. 450 mg globin A; 25 ml fractions; pH 1.9; IRC-50 column; interrupted urea gradient.

More recently we have found that 3 *M* urea can replace the 2 *M* solution as the initial eluting agent without causing any loss of the α chain. One may, after the initial phase of the procedure, shift directly to a urea gradient starting with the 3 *M* solution, or jump the urea concentration to approximately 4.0 to 4.2 before starting the gradient, as described above, the latter procedure shortening the total time of the run. Similarly, since the β peak and the material eluted with 8 *M* urea are identical on peptide analysis, one may switch over to 8 *M* urea as soon as it is apparent that the β peak is coming off the column.

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Protein determinations were carried out by the method of LOWRY *et al.*[13] on 0.2 ml aliquots. The presence of heme was ascertained by the benzidine method of CROSBY AND FURTH[14] on 0.5 ml samples. Approximate urea molarities were calculated by standard mathematical techniques utilizing the known initial pot volume and urea concentration and determining the molarity at any point after the addition of the specified amount of 8 *M* urea.

The isolated fractions were dialyzed against 8 to 10 changes of distilled water at 4° until urea was no longer detected by the diacetyl monoxime method[15]. Overnight dialysis versus cold running tap water resulted in the precipitation in the dialysis bags of considerable material which was poorly digested by trypsin and yielded inadequate fingerprints. Hence, this method was abandoned. Peptide analyses of the separated, lyophilized fractions were carried out by techniques described in a separate communication[16].

RESULTS AND DISCUSSION

Comparative elution curves of the α and β chains of human hemoglobin resulting from the two methods of separation are presented in Figs. 1, 2 and 3. In general, their characteristics are remarkably similar. The series of peaks, eluted by 2 or 3 *M* urea within the first 250 to 350 ml of effluent (fraction X in Figs. 1 and 2), strongly absorb U.V. light at 280 mμ. This material, however, does not react with ninhydrin, with Folin reagent or with benzidine and probably represents formic acid washed out of the column by the advancing buffer front. The α peaks of both Hgb A and Hgb F make their appearance at urea concentrations as low as 3.8 *M*, although maximal elution occurs at approximately 4.9 to 5.1 *M*. β chain elution starts at a urea molarity of

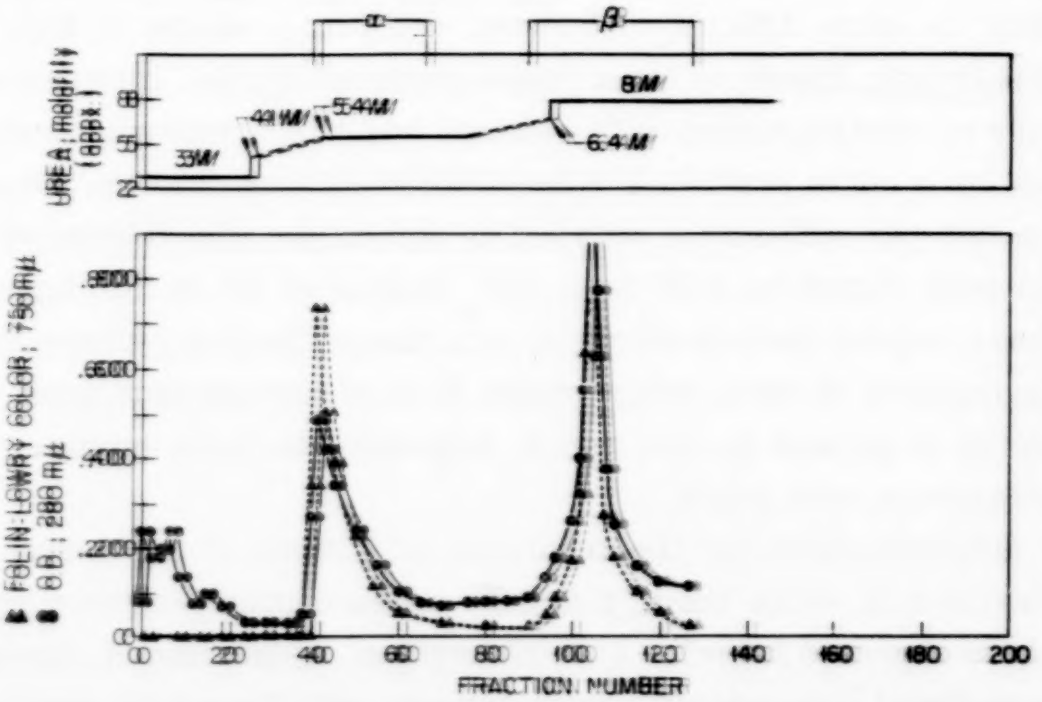

Fig. 3. Separation of the α and β chains of globin A by the interrupted gradient technique. 650 mg globin A; 25 ml fractions; pH 11.9; IRC-50 column; interrupted urea gradient. Also shown are the intensities of Folin-Lowry color and approximate urea molarities.

approximately 5.8 while maximal elution occurs in the range of 6.2 to 6.4 *M*. The γ chain characteristically is washed from the Amberlite resin at somewhat lower molarities, elution starting at 5.4 to 5.5 *M* with peak recovery at approximately 5.9 to 6.1 *M* urea. These figures are at considerable variance with those reported by HUISMAN AND SEBENS[17,18] who noted elution for all three chains at significantly higher molarities. We can offer no ready explanation for the inconsistencies noted, but it is possible that, because of the differences in techniques in our respective laboratories, such variations are more apparent than real.

Certain characteristics of the types of peaks obtained by the "interrupted" or "intermittent" gradient system deserve mention. The α and β curves may be separated by as great a distance as the operator desires by appropriate adjustment of the starting volumes of solution, provided one knows at approximately what molarity of urea maximal elution of the chains occurs. Furthermore, the sharpness of the graphed peaks provides an indication of the cleanness of separation—an abrupt rise and slightly slower decrease characterizing the elution of relatively uncontaminated peptide fragments. Since the gradient is interrupted just after the α peak is detected by the recorder, it is apparent that a urea molarity somewhat higher than indicated above (because of dead space and holdup volume) will "wash" the column until the gradient is resumed. Continuing the "wash" with the urea solution of constant molarity permits the curve to return almost to the base line before reinstituting the gradient for β chain elution. In the technique of WILSON AND SMITH, the "trough" between the two peaks remains considerably above the base line due to considerable mixing of the respective chains in this area. Visual inspection of the recording curve also permits one to assess the completeness of α chain removal from the resin. On resumption of the gradient, an additional small peak before the main β chain elutes or marked skewing of the curve of β chain elution suggests incomplete separation of the two polypeptide chains. Although rarely a problem in dealing with β chains, complete separation may be more difficult to achieve with the γ chains of Hgb F. Peptide patterns of the tryptic digests of the α chains prepared by the "interrupted" method reveal virtually no contamination with β chains, while only very slight contamination of the β chain by α chain peptides has been observed. Our data on the γ chain contamination is not yet sufficiently detailed to determine the degree of admixture.

The final peak eluted by 8 *M* urea, and designated IV in the figures, is highly pigmented, has a peptide pattern identical with that of the β or γ chains and probably represents aggregation of these polypeptides. It is of interest that most of the heme positive material is present in this region, although the total amount of benzidine reacting substances is very small.

Protein determinations by the technique of LOWRY *et al.*, reveal no reacting material in fraction X, while the α, β and IV peaks contain amounts equivalent, in general, to those expected from the U.V. absorption. As mentioned above, the benzidine positive material is associated almost entirely with the initial portion of fraction IV although, occasionally, traces may be seen in the early parts of the α and β fractions.

Following the separation, dialysis and lyophilization of the α and β chains as described above, approximately 60 to 65 % of the starting globin is recovered. In the technique of WILSON AND SMITH, approximately 30 % of the total recovery represents α chains, 25 % β chains, 10 % fraction IV and 35 % the mixed material between the α and β peaks. In the interrupted gradient method, the corresponding values are 40 to 45 % for α, 5 to 10 % for the intermediate zone, 30 to 35 % for β and 10 to 15 % for fraction IV.

SUMMARY

A modification of the gradient elution method of WILSON AND SMITH for the preparation of the individual polypeptide chains of human globin is described. The chief advantage of the modified procedure relates to the improved separation between the α and β or γ chains.

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CHROMATOGRAPHIC STUDY OF HUMAN SERUM BY GEL FILTRATION

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(Received December 27th, 1960)

By means of cross-linked dextran polysaccharide, it has become possible to separate substances of different molecular size by gel filtration[1-3]. The dextran network has polar properties due to the high content of hydroxyl groups and has a low content of ionized groupings[2]. The degree of cross linkage determines the minimum size of particle excluded from the interstices of the gel grains. These are therefore able to pass through the column at a rate greater than that of molecules able to enter the grains. This method of separation is applicable to ionized as well as nonionized solutes.

Solutes passing through a gel column can be characterised by a partition coefficient (K_D)[2] between the external aqueous phase (void volume V_0) and the internal aqueous phase of the grains (V_i). The internal aqueous phase is determined as the product of the water regain of the gel and the weight of dry material used. When the total elution volume (V_e) for a solute is known, the partition coefficient may be expressed as:

$$K_D = \frac{V_e - V_0}{V_i} \qquad (1)$$

A solute of low molecular weight can freely diffuse into the grains and has a K_D of about 1. However, since part of the inner volume (V_i) is bound to the polysaccharide matrix as water of hydration, thus restricting the size of the interstitial system, small solute particles usually show a partition coefficient of about 0.8, indicating nonrestricted diffusion. Substances partially excluded from the gel grains because of molecular size have a K_D value between 0 and 1.

Studies by GELOTTE[2] and PORATH[4] have shown sorption of solutes high in aromatic amino acid, as well as basic amino acids such as histidine, when the eluant was distilled water. When the eluant contains electrolyte, this sorption phenomenon for basic amino acids disappears. These effects are ascribed to small amounts of ionized carboxylic groups in the bed material reacting with the positively charged groups of the solute[2,4]. Such materials show a K_D value of > 1.0.

The special qualities of this system make possible the rapid separation of proteins from solutes of lower molecular size, as well as from their electrolyte vehicle. At the same time this permits transfer of large molecules such as proteins from one solvent to another in a single passage through the column.

At least one further application of the unique conditions produced by gel filtration appeared possible. Implicit in the separation of large molecular weight protein from its solvent system is that the solubility of the protein in the equilibrating buffer be the same as that of the solvent used to load the column. If the proteins are less soluble in the eluant than the original solvent, the protein would be restricted in its flow down the column to the front containing the original solvent system. In a mixture of proteins of different solubilities in this regard separation would be effected. If the differential solubility is determined by an eluant of low ionic strength, in contrast to the original protein solvent of higher ionic strength, the electrolyte dilution due to diffusion into and out of the gel grains could be controlled by the addition of electrolyte to the protein mixture before loading the column.

Normal human serum provides a mixture of proteins, some of which are insoluble in media of low ionic strength (euglobulins) in contrast to those soluble in media of low ionic strength (pseudoglobulins). Certain human sera obtained from patients with diseases such as rheumatoid arthritis and macroglobulinemia are particularly rich in euglobulins, in part due to their increased macroglobulin content.

METHOD

A column, 29 cm long and 2 cm in diameter, was used for 2 ml samples of serum, and was run at room temperature.

The gel material used in the present study, Sephadex G-25, excludes materials of greater than 2000 to 3000 molecular weight from the grains, is insoluble in water and salt solutions and is stable in alkaline solutions and weak acids. The water regain is 2.7 g/g of dry weight.

Sephadex G-25, 20.7 g, was washed in distilled water and permitted to settle three times to remove fine particles. It was then poured into the column and permitted to settle by gravity. The column was equilibrated with 0.02 *M* Na phosphate buffer, pH 8, and mounted over an automatic fraction collector. The eluate for all studies was the same buffer used for initial equilibration of the column.

Determination of void volume

The void volume was determined by the application of 0.1% ferritin solution to the column and then elution with the same buffer system to be used in the protein separation. The void volume measured to the point of the first appearance of ferritin off the column was 40 ml.

Preparation of sera

Sera were from normal subjects and from patients with rheumatoid arthritis in which rheumatoid factor, a macroglobulin having the solubility characteristics of an euglobulin, could be measured by a hemagglutination system using tannic-acid treated sheep erythrocytes coated with commercial preparations of normal human γ-globulin (F-II hemagglutination test)[5].

Sera were either dialyzed against 1 N NaCl prior to gel filtration, or had NaCl added to produce a final ionic strength equivalent to 0.85.

The protein content of elution fractions was determined as extinction values at 280 mμ using a Beckman DU spectrophotometer. The electrolyte content of elution fractions was determined by conductivity measurements with values expressed as 10^{-4} mho. The electrophoretic pattern of the elution fractions was determined using an agar supporting medium on glass slides (pH 8, barbital buffer, 250 V, 30 min).

RESULTS

The pattern for the protein content and conductivity of the fractions obtained using normal human serum are shown in Fig. 1. Four peaks designated I, II, III and IV are recorded. Peak I emerges in a medium having the conductivity of the buffer used for equilibration of the column. Peak II emerges just ahead of the peak of conductivity but has components extending to the peak of salt content. Peaks III and IV, having K_D values of 1.4 and 2.13 respectively, are materials capable of sorption to the column matrix.

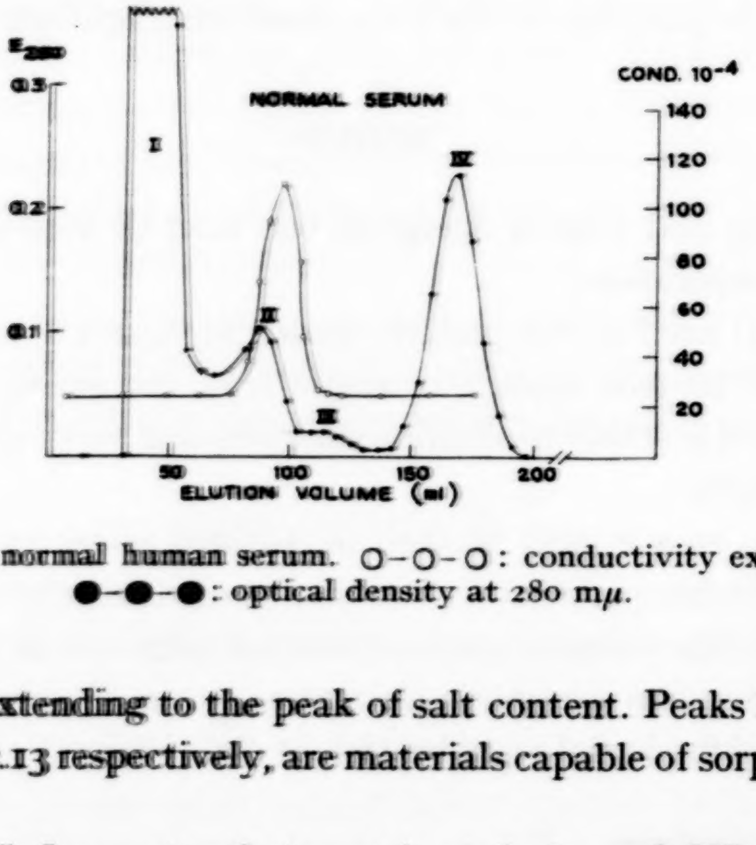

Fig. 1. Gel filtration of normal human serum. ○-○-○ : conductivity expressed as 10^{-4} mho; ●-●-● : optical density at 280 mμ.

After further study by spectrophotometric analysis, peak III showed a maximum value at 255–270 mμ and peak IV at 290 mμ. This, together with failure of the material to precipitate on the addition of 10% trichloracetic acid, indicated we were dealing with predominantly nonprotein material. As shown in Fig. 2, the pattern produced by gel filtration of normal serum after dialysis of the serum against 1 N buffered saline, pH 8, demonstrates the dialyzable nature of the nonprotein material constituting peaks III and IV.

In order to demonstrate that the migration of the protein of peak II is determined by solubility characteristics, peak II was re-run on a Sephadex column which was equilibrated with 0.15 M phosphate buffer, pH 8. Elution of peak II with the same buffer revealed an elution volume similar to that of peak I (Fig. 3).

The electrophoretic pattern of the proteins found in peaks I and II of normal

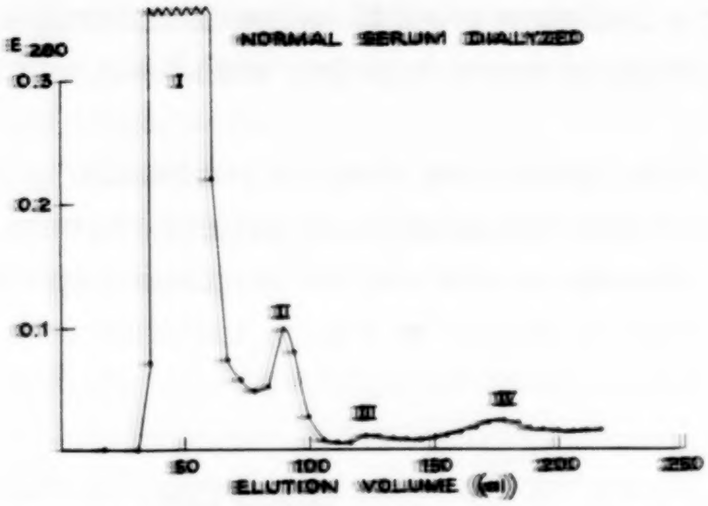

Fig. 2. Gel filtration of normal human serum after dialysis against 0.15 *M* phosphate-buffered saline, pH 8, 24 h at 4°.

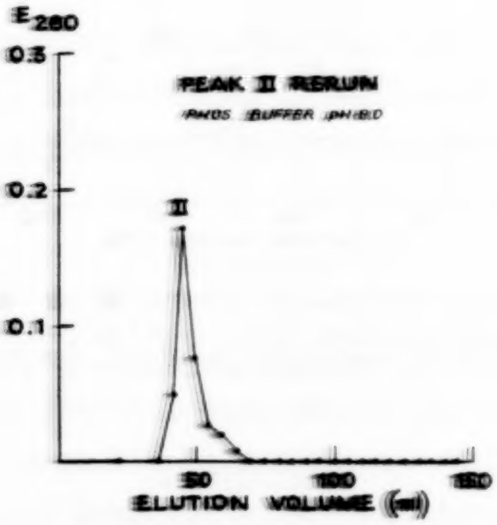

Fig. 3. Re-run of peak II obtained from Sephadex column equilibrated and eluted with 0.15 *M* phosphate-buffered saline, pH 8.

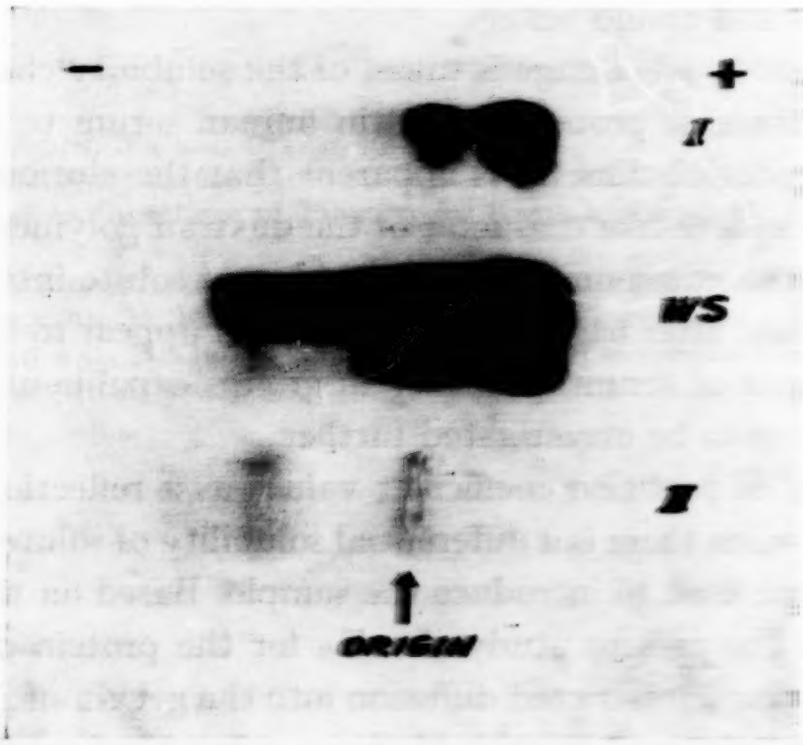

Fig. 4. Electrophoretic pattern: top, peak I; center, whole serum; bottom, peak II. γ-Globulin in peak II is in the γ_1 position.

serum are shown in Fig. 4 and show peak II to consist entirely of γ-globulin and peak I to contain the other serum proteins, together with what is presumed to be pseudoglobulin γ-globulin.

The concentration of a particular class of euglobulin γ-globulin in peak II is shown by gel filtration of sera obtained from patients having rheumatoid arthritis. The rheumatoid factor content of the elution fractions expressed as $\log_2$ reciprocal F-II hemagglutination titer is shown in Fig. 5, together with the elution and conductivity pattern.

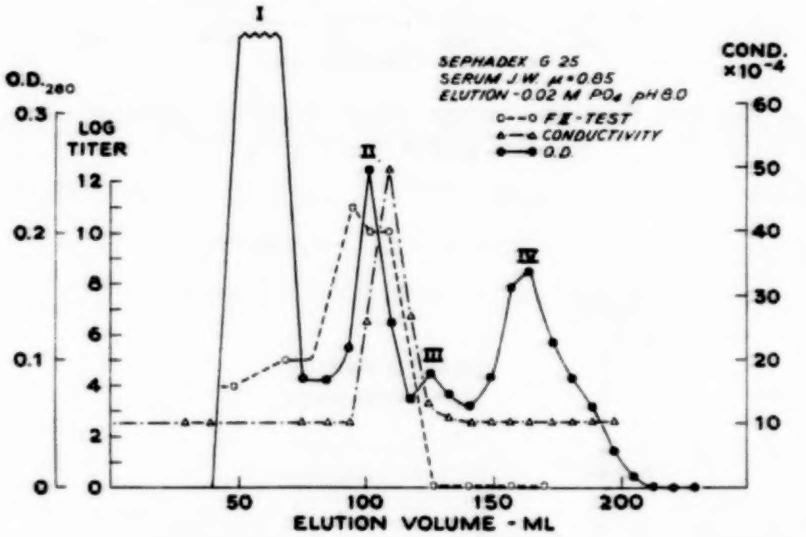

Fig. 5. Gel filtration of nondialyzed rheumatoid serum. ●–●–● : optical density; -△---△-: conductivity; ○--○ : $\log_2$ hemagglutination titer. NaCl added to serum prior to addition to the column of final ionic strength of 0.85. Elution with 0.02 *M* phosphate-buffered saline, pH 8.

DISCUSSION

The initial application of the technique of gel filtration to the separation of materials of markedly different molecular size has been of outstanding value in accomplishing in a short time the effects of desalting by dialysis as well as the separation of mixtures of proteins, peptides and amino acids[4].

In the present study advantage is taken of the solubility characteristics of some members of the mixture of proteins found in human serum to effect separation of euglobulins and pseudoglobulins. It is apparent that the elution characteristics are not dependent on the sieve-like character of the dextran polymer since all the serum proteins far exceed the minimum size for diffusion of solute into the gel interstices. The two peaks obtained after passage of the salt front appear to be due to nonprotein dialyzable constituents of serum, probably in groups capable of partial sorption on the dextran. These are to be investigated further.

The designation of partition coefficient values as a reflection of size of particle becomes impossible when there is a differential solubility of solute in the equilibrating buffer and the solvent used to introduce the sample. Based on the characteristics of the column used in the present study the K_D for the protein of peak II in Fig. 1 would be 0.7, indicating unrestricted diffusion into the gel grains. This is certainly not true for the proteins contained in peak II. Since it has been established that peak II will be eluted with peak I when the equilibrating buffer has sufficient electrolyte content to allow unrestricted motion through the external aqueous phase, it follows that a meaningful partition coefficient can be derived for such proteins only when the solvent furnishes no restriction to flow.

The concentration of rheumatoid factor activity in peak II confirms the euglobulin character of this class of γ-globulin. Further purification of rheumatoid macro-γ-globulin from peak II by anionic-exchange chromatography is described in another paper[6].

It would appear that the principle of gel filtration, combined with the effects of the differential solubility of the solute in the loading solvent, compared to the equilibrating and eluant solutions, allows separation of mixtures of proteins of similar size but differing solubility characteristics.

ACKNOWLEDGEMENTS

Supported in part by a grant (A-1229) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and by funds from the Northern California Chapter of the Arthritis and Rheumatism Foundation.

SUMMARY

Gel filtration, using polymerized dextran granules in a chromatographic system, has been used to separate two classes of human serum proteins, both of which are unable to enter the gel matrix. The euglobulin proteins are restricted in their flow to a front containing the solvent used to load the serum on the column, while serum pseudoglobulins separate due to their solubility in the low ionic strength equilibrating buffer.

The concentration of one class of serologically measurable serum euglobulin (rheumatoid factor) has been demonstrated.

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IDENTIFICATION, ESTIMATION AND PREPARATION OF FATTY ACIDS BY CIRCULAR PAPER CHROMATOGRAPHY

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(Received December 21st, 1960)

INTRODUCTION

During recent years a considerable amount of attention has been devoted to the development of rapid methods for the identification, isolation and quantitative estimation of fatty acids in biological materials. Reversed phase paper chromatography has been applied for the identification and estimation of fatty acids, using polarographic determination of copper[1], photometric estimation of copper as the dithiooxamide complex[2], microphotometric estimation of copper as a salt of rubeanic acid[3], or colorimetric estimation of copper as a salt of tetraethylthiuram disulphide[4]; in all these methods copper acetate was made to react with the fatty acids. The "Critical Pairs" of fatty acids have been analysed before and after hydrogenation of the mixture of fatty acids and before and after oxidation with alkaline permanganate[2], and small amounts of saturated acids occurring along with large amounts of unsaturated acids have been identified after oxidation of the unsaturated acids with peracid[5]. In the qualitative analysis of fatty acids by other methods various reagents have been used, *e.g.* bismuth sulphide[6], complex of mercury with *s*-diphenylcarbazide[5], and, specifically for unsaturated acids, osmium tetraoxide[7]. The use of high temperatures has been tried for the separation of higher saturated fatty acids, particularly those with chain lengths from twenty onwards[8].

All these methods either require special equipment or are not sufficiently rapid; moreover, the various methods differ only in regard to the reagents employed. Recently we have developed a comparatively simple method for the identification, isolation and quantitative estimation of saturated as well as unsaturated fatty acids, using the circular paper chromatographic technique and combining two of the conditions suggested in the literature[5,8]. The possibility of preparing the fatty acids was also examined. The results of these studies are briefly described in this paper.

EXPERIMENTAL

Quantitative estimation of fatty acids

The saturated straight chain fatty acids C_{18} and C_{16}, C_{14} and C_{12}, were separated from their corresponding critical components by the peracid procedure[5], using a 24 cm circular Whatman No. 3 paper impregnated with 10% liquid paraffin. The C_{18} acid and the three critical pairs from C_{16} to C_{12} were identified after developing the chromatogram in 90% aqueous acetic acid and then treating it with mercuric acetate and *s*-diphenylcarbazide[5]. The fatty acids were estimated by extracting the mercury colour complex in a freshly distilled 1:1 mixture of methyl alcohol and toluene. As the colour complex had an absorption maximum at 540 $m\mu$, it was estimated colorimetrically by using a Klett-Summerson photoelectric colorimeter with 530 $m\mu$ green filter. All the seven fatty acids gave a linear relationship between 5 μg and 60 μg. The colour was stable even at the end of 4 hours. The colour faded, however, in presence of the smallest amount of moisture.

With this circular paper chromatographic technique the fatty acids were separated very rapidly: in 2 1/2 hours the solvent travelled a distance of 10 cm and gave a very clear separation, the R_F values of the fatty acids differing by 0.1. In this method only linoleic acid gave a rose coloured mercury–*s*-diphenylcarbazide complex, whereas the rest of the fatty acids gave a purple colour. The linoleic acid was found between palmitic and myristic acid, more towards myristic acid.

This technique has been successfully used for the analysis of the fatty acids in biological materials such as *Aspergillus niger*, *Malbranchia pulchella*, activated sludge, sesame oil, mustard oil, and goat liver. The results are given in Table I.

TABLE I

FATTY ACID COMPOSITION OF VARIOUS BIOLOGICAL MATERIALS

(Results expressed as percentage)

Material	*Saturated fatty acids*				*Unsaturated fatty acids*		
	Lauric	*Myristic*	*Palmitic*	*Stearic*	*Linolenic*	*Linoleic*	*Oleic*
1. *Aspergillus niger*	—	—	28.3	22.0	—	31.3	15.1
2. *Malbranchia pulchella**	—	—	30.4	13.5	—	32.6	21.2
3. Activated sludge**	1.4	2.3	20.2	15.6	1.1	35.2	20.1
4. Sesame oil	—	—	8.5	5.3	—	35.2	48.1
5. Mustard oil***	—	—	1.0	—	4.1	8.7	33.0
6. Goat liver	—	—	25.4	30.3	—	12.3	30.2

* *Malbranchia pulchella* is a thermophilic strain from Indian compost; it was isolated by Miss M. PREMA BAI and Dr. P. L. NARASIMHA RAO of this Department.

** Activated sludge is a sewage sludge formed under aerobic conditions, which shows intense microbial activity. In this sludge the presence was observed of traces of three saturated fatty acids with R_F values lower than that of stearic acid, which still separate at room temperature.

*** The amount of erucic acid present in the mustard oil was 53.2% (determined by difference).

— Not present.

Isolation and preparation of the fatty acids

Reversed phase circular paper chromatography was used for the isolation and preparation of fatty acids. Forty mg of a commercial linoleic acid (containing 46.5% linolenic, 19.6% linoleic, 24.8% oleic and 8.2% palmitic acid) was spotted at the centre of the paraffin-impregnated paper. The mixture of the fatty acids was spread over a circle of 1.5 cm diameter. This was developed thrice with 90% aqueous acetic acid and exposed to iodine vapours whereby a clear-cut separation of the unsaturated fatty acids occurred, showing three bands. A photograph of the chromatogram is shown in Fig. 1. The individual bands were cut out, eluted in ethyl alcohol and neutralised with *N*/10 sodium hydroxide using phenolphthalein as indicator. The alcoholic solution was made slightly alkaline and the unsaponifiable, *i.e.* liquid paraffin, was extracted with ethyl ether after evaporation of the alcohol and dilution of the soap solution with water. The soap solution was then acidified and the liberated fatty acids were extracted with ethyl ether. The ether solution was then washed free of mineral acid and after evaporation of the ether the weight of the fatty acids was determined. The recovery on the whole was 75%, the amounts of the fatty acids recovered being: linolenic, 12 mg; linoleic, 6 mg; and oleic *plus* palmitic 12 mg (the amounts of the two last-mentioned acids, estimated by chromatography were 9.35 mg oleic and 2.70 mg palmitic acid). The linolenic acid had no impurities, whereas linoleic acid contained traces of linolenic acid, and oleic acid contained traces of linoleic acid. When twice the amounts of fatty acids were spotted the number of developments was increased twofold, *i.e.* to six. When 40 mg of a mixture containing only saturated fatty acids was spotted, the solvent did not move because of the solidification of the fatty acids and consequent blocking of the pores of the paper. When, however, saturated fatty acids were dissolved in an unsaturated acid in equal amounts (total 40 mg) and spotted, the solvent moved normally, and after four developments a clear-cut separation of the fatty acids was obtained. The yields were 15 mg of oleic acid and 16 mg of stearic acid from a mixture of 20 mg each of oleic acid and stearic acid. The recovery on the whole was 78%.

Fig. 1. Chromatogram of the unsaturated fatty acids from commercial linoleic acid, with three bands corresponding to: 1. Linolenic acid; 2. Linoleic acid; 3. Oleic acid.

The above procedure might be useful for the isolation of any new fatty acids present in biological materials.

Identification of higher saturated fatty acids

High temperatures up to 85° have been used for the chromatographic separation of higher saturated fatty acids from C_{20} onwards[8]. In the present study the red seeds of ***Adenanthera pavonina***, which are known to be a good source, particularly of lignoceric acid[9], were used for analysis. When this material was analysed chromatographically at room temperature (25°), only two bands for unsaturated fatty acids corresponding to oleic and linoleic acid were observed and three bands for the saturated acids corresponding to myristic, palmitic, and stearic acid. However, a purple streak from the starting point also appeared, indicating the presence of lignoceric acid. When the chromatogram was run at 55° and then exposed to iodine vapours, it was possible to observe five bands representing five unsaturated acids. Of these, three bands were prominent and the other two rather faint. A photograph of this chromatogram is given in Fig. 2, in which the two faint bands are not visible.

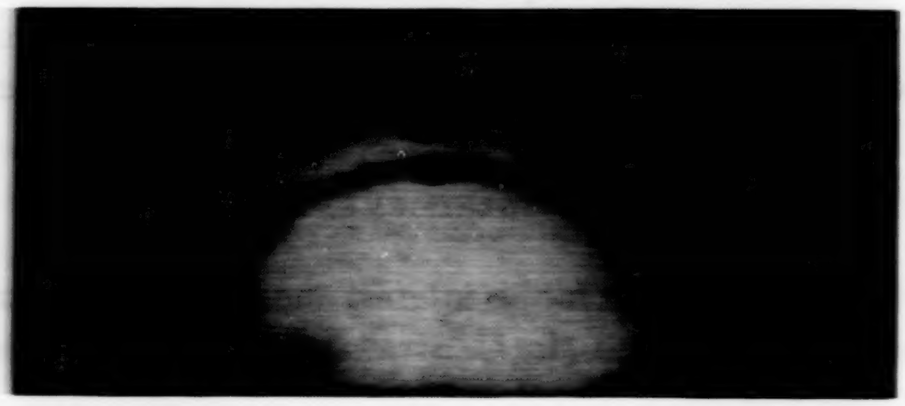

Fig. 2. Chromatogram (run at 55°) of the unsaturated fatty acids from the seeds of *Adenanthera pavonina*, with three bands (two other bands are not visible) corresponding to: 1. Linoleic acid; 2. Oleic acid; 3. Not identified.

The unsaturated acids in the seed material were destroyed by the peracid procedure[5], and another chromatogram was run for the analysis of saturated fatty acids at 55°. A photograph of this chromatogram is given in Fig. 3, in which bands for eight saturated fatty acids can be seen.

Fig. 3. Chromatogram (run at 55°, after peracid treatment) of the saturated fatty acids from the seeds of *Adenanthera pavonina*, with eight bands corresponding to: 1. Myristic acid; 2. Palmitic acid; 3. Stearic acid; 4 to 8. Not identified.

DISCUSSION

The circular paper chromatographic method described above for the quantitative estimation of fatty acids seems to be fairly simple and rapid. The time taken for the determination did not exceed 5 hours. The method is particularly useful for the analysis of the "Critical Pairs" of fatty acids; for such an analysis the method available up to the present is hydrogenation of the acids and oxidation with alkaline permanganate, which is somewhat tedious[2]. Another attractive feature of the circular paper chromatographic technique is its possible use as a preparative method also.

With regard to the use of high temperatures for the identification of higher saturated fatty acids, the circular paper chromatographic technique also offers advantages. For instance, by the earlier method[9], only two unsaturated and four saturated acids were identified in the seeds of *Adenanthera pavonina*, whereas with the technique described here, it was possible to identify five unsaturated and eight saturated fatty acids in the same material. Thus circular paper chromatography could be used for a more sensitive or effective analysis of the fatty acids in biological materials.

ACKNOWLEDGEMENTS

We are grateful to Dr. S. C. PILLAI for his interest and advice. Our thanks are also due to Dr. P. L. NARASIMHA RAO and Mr. U. SITARAMA ACHARYA for their suggestions.

SUMMARY

A comparatively simple and rapid method for the identification, estimation and preparation of fatty acids has been developed, using reversed phase circular paper chromatography. The method is also suitable for the analysis of "Critical Pairs" of fatty acids and for the preparation of fatty acids. Further, when used at a higher temperature, the method is more sensitive in revealing the presence of even traces of higher fatty acids in the seeds of *Adenanthera pavonina*.

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PAPER CHROMATOGRAPHY OF INORGANIC IONS IN NITRATE MEDIA

III. SEPARATION OF CALCIUM, STRONTIUM, BARIUM AND RADIUM

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(Received December 19th, 1960)

The separation of the alkaline earth elements by paper chromatography in nitric acid media was investigated by ELBEIH *et al.*[1], who used collidine as organic solvent, and by NAKANO[2], who used a mixture of HCl and HNO_3 in methyl alcohol.

The alkaline earths associate with the nitrate ion to form complexes with different stabilities[3]. Nitrate media should therefore be useful for chromatographic separations of these elements, particularly at high nitrate concentrations as was observed in other systems[4,5].

EXPERIMENTAL

Small aliquots of about 0.02 ml of the samples were applied to a strip of Whatman No. 1 paper, which was placed in a closed chamber for ascending development. The eluting solvents were treated beforehand with appropriate nitrate solutions and development was carried out at room temperature for about 16 hours.

The separation of Ca–Sr–Ba was investigated with about 0.2 mg of each cation. Calcium was detected by spraying an alcoholic solution of 8-hydroxyquinoline in ammoniacal medium; the spots obtained became fluorescent on irradiation with ultraviolet light. Strontium and barium were identified with potassium rhodizonate, red spots being obtained.

Separations of barium and radium were investigated at the tracer level by using carrier-free ^{140}Ba ($\beta, \gamma, T_{1/2} = 12.8$ days) and ^{228}Ra (MsTh I, β, γ, 6.14 years) as isotopes. Both isotopes were obtained from the Atomic Energy Research Establishment, Harwell, England.

After development the strip of paper was scanned with a Geiger-Müller counter. The measurements were made some days after the development in order to attain the transient equilibrium between the tracers and their radioactive descendents, which are ^{140}La (β, γ, 40 h period) and ^{228}Ac (β, γ, 6.13 h period). The separations of ^{140}Ba–^{228}Ra were also checked by measurement of the γ ray spectra of the isotopes in a single channel spectrometer with Na I (Tl) crystal.

RESULTS

(a) Separation of Ca–Sr–Ba

Table I gives the R_F values obtained with various organic solvents with different $LiNO_3 + HNO_3$ concentrations. It can be seen that the differences between the R_F's of these elements are larger with the more concentrated nitrate solutions. The R_F's are strongly dependent on the nature of the organic solvent. The value of the R_F was found to decrease with increasing molecular weight of the alcohols investigated. Better separations were obtained with lower alcohols, such as ethyl alcohol, or with diethyl ketone.

TABLE I

Organic phase	*$LiNO_3$ molarity*	*HNO_3 molarity*	R_F		
			Ca	*Sr*	*Ba*
Amyl alc.	7	8	0.043	0	0
Butanol	9	0.5	0.182	0.062	0
Butanol	7	8	—	0	0
Butanol	7	2	—	0.033	0
Propanol	9	0.5	0.8	0.6	0.402–0.04
Propanol	7	2	0.71	0.47	0.33 –0.074
Propanol	7	—	0.648	0.495	0.33 –0
Propanol	4	0.5	0.616	0.385	0.19 –0
Isopropyl alc.	7	2	—	—	0.212–0
Ethanol	7	2	0.7	0.425	0.233–0.039
Ether	7	2	—	—	0
Acetone	3	2	0.688	0.537	0.356–0.073
Acetone	3	0.5	0.575	0.540	0.406–0.066
	$Al(NO_3)_3$				
Butanol	Satur.	0.5	0.142	0.056	0
Propanol	Satur.	0.5	—	0.18	0.094–0

(b) Double spot formation with barium

As can be observed in Table I, barium nitrate yields a double spot when the development is carried out with low molecular weight solvents. Similar results were obtained by using solvents saturated with $Al(NO_3)_3$ instead of $LiNO_3$.

Since the formation of double spots was observed with different samples of $Ba(NO_3)_2$ and also when acid-washed paper was used it cannot be attributed to the presence of impurities in the solution or in the paper.

The formation of multiple spots of a given element has been observed by several authors in various systems[4-6]. The existence of more than one spot is generally attributed to the formation of stable complexes of the element in the media investigated. This explanation, as well as others, for the formation of multiple spots has been discussed in a recent review[7].

A remarkable aspect of the double spot formation of Ba in nitrate media was observed when the concentration of the cation Ba^{2+} was varied. The chromatograms reproduced in Fig. 1, obtained with a mixture of propanol (80 %) and an aqueous solution (20 %) of $LiNO_3$ (7 *M*) and HNO_3 (2 *M*), show that the double spot formation is a function of the initial concentration of barium. With amounts of barium higher than $2 \cdot 10^{-4}$ mg two peaks are observed ($R_F = 0$ and $R_F = 0.4$). At about 10^{-4} mg the two peaks join and a "comet" is observed. With smaller amounts of barium only a single peak is observed ($R_F = 0.4$). The concentration of barium in the peak is thus dependent on the initial concentration of the cation.

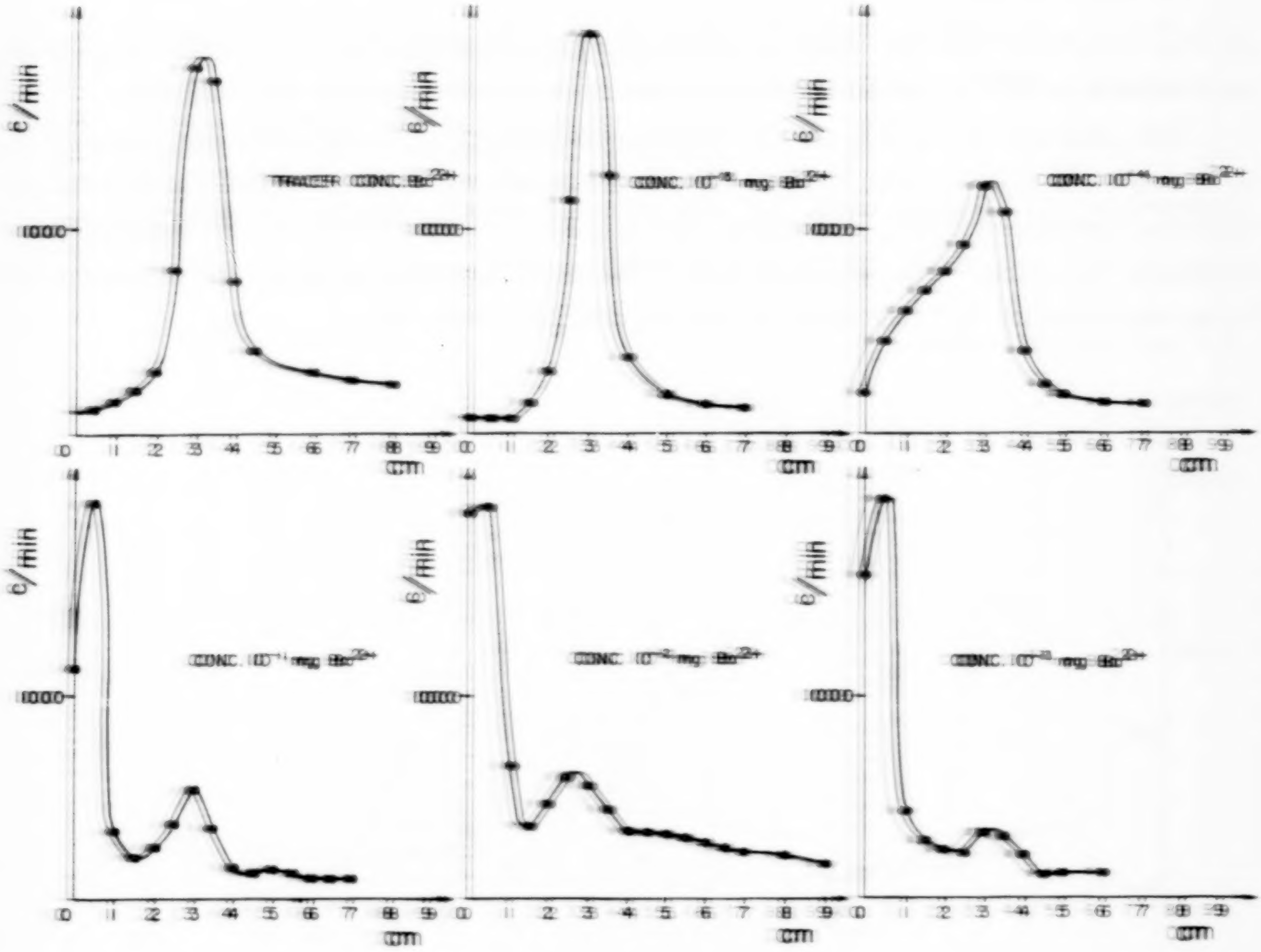

Fig. 1.

This behaviour cannot be explained by assuming that the double spots are due to the formation of barium complexes possessing different R_F values, such as:

$$\text{Ba}^{2+} + x\,\text{NO}_3 \rightleftharpoons \text{Ba(NO}_3)_x^{2-x} \qquad (1)$$

The equilibrium constant for this reaction is:

$$K = \frac{[\text{Ba(NO}_3)_x^{2-x}]}{[\text{Ba}^{2+}]\,[\text{NO}_3^-]^x} \cdot \Gamma \qquad (2)$$

where Γ is the activity coefficient ratio of the species. At constant $[\text{NO}_3^-]$, Γ can be assumed as constant and we have:

$$\frac{[\text{Ba(NO}_3)_x^{2-x}]}{[\text{Ba}^{2+}]} = K' \qquad (3)$$

The relation between the concentration of barium in the two peaks should thus be independent of the initial concentration of the cation, if only mononuclear barium nitrate complexes are formed. We are of the opinion that further investigations are required in order to explain the anomalies observed in the chromatographic behaviour of barium.

(c) *Separation of Ba–Ra*

As was observed with the other systems, the chromatographic separation of barium and radium in HNO_3 media with propanol as organic solvent is not efficient.

The addition of $LiNO_3$ to the HNO_3 solution greatly improves the separation, as is shown in Fig. 2. This chromatogram was obtained with propanol (80 %) and an aqueous solution of HNO_3 (2 *M*) and $LiNO_3$ (4 *M*). Trace amounts of the elements were employed by using ^{228}Ra (MsTh I) and ^{140}Ba as radioactive isotopes. All other conditions were similar to those used in the separation Ca–Sr–Ba.

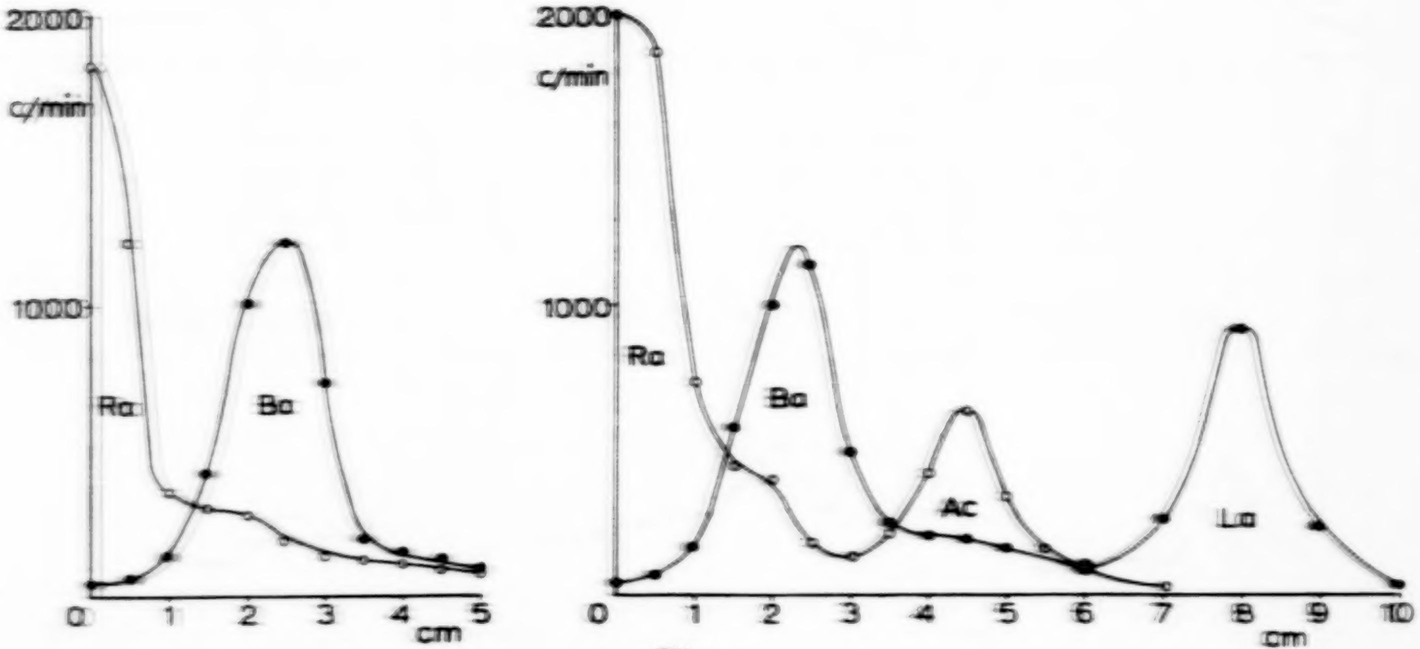

Fig. 2.

It is important to observe that the separation cannot be effected with larger amounts of barium ($> 10^{-7}$ g), since this element gives two spots, the first one with $R_F = 0$, equal to that of radium in trace amounts.

Since the R_F values of Ac ($R_F = 0.25$) and La ($R_F = 0.47$) differ considerably from those of Ra and Ba respectively, the method is useful for the purification of these radioactive elements by paper chromatography, as is illustrated in Fig. 2.

ACKNOWLEDGEMENT

This work was supported by the Conselho Nacional de Pesquisas.

SUMMARY

The separation of Ca–Sr–Ba was achieved by paper chromatography using concentrat-

ed nitrate solutions with propanol as solvent. Ba yields double spots with some organic solvents.

The method enables a separation of Ba and Ra in trace amounts and is useful for the radiochemical purification of ^{140}Ba and ^{228}Ra sources.

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Notes

Chromatographic estimation of stearoptene in Indian rose oils

The stearoptene content of a sample of rose oil is one of its imporant characteristics[1]. The United States Pharmacopoeia gives a limiting test[2] for minimum stearoptene content. Estimation[3] of stearoptene is usually carried out by chilling a solution (of 5 g) of the oil in 75 % ethyl alcohol, filtering and weighing the separated stearoptene. The method is not convenient in tropical countries and sufficient care has to be taken during filtration and subsequent estimation of stearoptene. Another disadvantage from which the method suffers is that a large quantity (5 g) of the oil is required for estimation. It was considered worthwhile to develop a simpler method for estimation of stearoptene using only a small quantity of the oil.

The stearoptene of Indian rose oils is composed of paraffin hydrocarbons. These can be successfully separated from the oil by chromatography over alumina. Fig. 1 shows the elution curve for 1 g each of three different types of Indian rose oils. It may be observed that 65 c.c. of the eluant carries away all the stearoptene (A). The product (B), which is present in negligible quantity, is eluted only after 80 c.c. of eluant has been collected. In the actual procedure 50 g grade I (Brockmann).

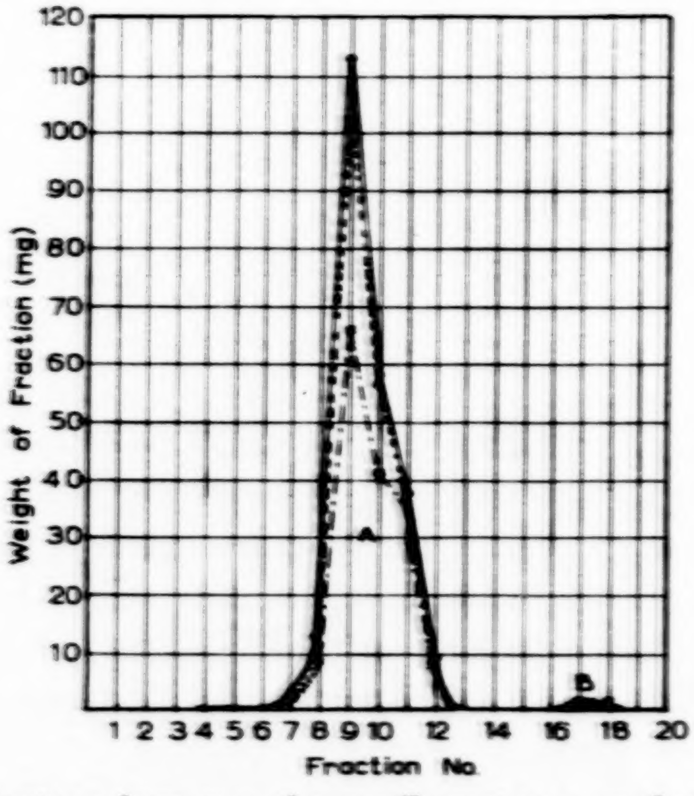

Fig. 1. Elution of stearoptene from 1 g of rose oil over 50 g grade I alumina. Volume of each fraction: 5 c.c. —o—o— oil of *Rosa damascena*; – – – · – – – · – – oil of *Rosa bourbonica*; · — × — · — × — · oil of rose teplitz.

alumina is packed in a column of 18 mm diameter. A solution of 1 g of oil is adsorbed at the top of the column and eluted with hexane. 70 c.c. of the eluant is collected and evaporated in a tared flask. From the weight of the residue, the percentage of stearoptene in the oil may be calculated.

The method was verified by estimation of stearoptene in a number of samples of Indian rose oils by the conventional as well as the above chromatographic method. The results were found to compare favourably, as shown in Table I for one representative experiment with each of the three different varieties of rose oils investigated.

TABLE I

Oil sample	*Stearoptene content*	
	Conventional method %	*Chromatographic method* %
1. *Rosa damascena*	23.5	23.2
2. *Rosa bourbonica*	22.1	21.8
3. Rose teplitz	16.3	16.1

Further verification was carried out by mixing stearoptene (isolated from rose oil) and eleaoptene (stearoptene-free oil) in different known proportions and estimating the stearoptene content of the mixture by the chromatographic method. The results were found to agree within 0.4% (Table II).

TABLE II

Oil sample	*Stearoptene content*	
	Actual content %	*Chromatographic estimation* %
1. *Rosa damascena*	15.2	15.0
	30.1	29.8
	45.1	45.0
2. *Rosa bourbonica*	15.2	14.8
	30.3	30.2
	45.2	45.1
3. Rose teplitz	15.0	14.7
	30.2	30.3
	45.2	49.9

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Received April 10th, 1961

The adsorption of thorium on a strong base anion exchange resin from nitrate media

The adsorption of thorium on strong base anion exchange resins from HNO_3 solutions has been examined by various workers[1-3]. Thorium is strongly adsorbed in concentrated HNO_3 (distribution coefficient K_D* reaches a maximum value of 200 in 7–8 M HNO_3), shows rapid decrease of K_D with decreasing HNO_3 concentration below 6 M HNO_3 and has negligible adsorption at low HNO_3 concentrations. Since the distribution coefficients of several elements (uranium[4,5], rare earths[6,7] and bismuth[8]) are significantly higher in nitrate solutions of low acidity (*e.g.* $LiNO_3$, NH_4NO_3, $Mg(NO_3)_2$ and $Fe(NO_3)_3$ solutions) than in HNO_3 solution, the adsorption of thorium from nitrate solutions of low acidity has been examined. Since the author was concerned with the processing of Al–Th, Ca–Th and Li–Th mixtures, aluminium, calcium and lithium nitrate solutions were selected for study.

A strongly basic anion exchanger of the polystyrene–divinylbenzene type (Deacidite FF, mesh size 100 to 200) with moderate cross-linkage (7–9 % D.V.B.) was used. Initially in the chloride form, it was converted to the nitrate form by treatment in a column with 2 M NH_4NO_3 solution until the silver nitrate test for chloride was negative. The resin was then washed with deionised water and air dried.

All reagents were C.P. or reagent grade.

Adsorbabilities were determined at room temperature (23 to 25°) by the batch equilibrium method. Weighed amounts of resin were shaken with a known volume of solution for 12 h. (Example: 0.25 g resin + 20 ml 0.2 $M$$LiNO_3$ solution containing 40 μg Th.) All solutions were maintained at pH 1.9 by the addition of a small amount of 1.0 M HNO_3 to avoid complications by possible hydrolytic reactions. From analysis of the thorium contents of the solution phase before and after equilibration, distribution coefficients K_D were computed. Thorium was determined spectrophotometrically with thoron[9].

The adsorption of thorium on Deacidite FF (NO_3 form) from $Al(NO_3)_3$, $Ca(NO_3)_2$ and $LiNO_3$ solutions of pH 1.9 at 23–25° is shown in Fig. 1. For comparison, CARSWELL's[2] anion exchange data of thorium in HNO_3 are included in Fig. 1.

It is seen from Fig. 1 that the distribution coefficient of thorium is increased if HNO_3 is replaced by nitrate solutions of low acidity. Adsorption was found to increase with the nature of the cation of supporting nitrate solution in the order

$$H < Ca < Li < Al$$

DANON[10] recently measured the adsorbability of thorium on Dowex-1 (NO_3 form) from 1.55–8.44 M $LiNO_3$. All solutions were 0.07 M in HNO_3. Thorium adsorbability (K_D) increased from 13 in 1.5 M $LiNO_3$ to 20,000 in 8.4 M $LiNO_3$.

Interestingly, the extraction of thorium, uranium and rare earths by tributyl phosphate and ether from nitric acid solution is favoured by the presence of inorganic nitrates[11-15]. In the case of tributyl phosphate extraction of uranium[12], the salting out effect of inorganic nitrates was most marked at low acidities.

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* $K_D = \dfrac{\text{Concentration of element/g resin}}{\text{Concentration of element/ml solution.}}$

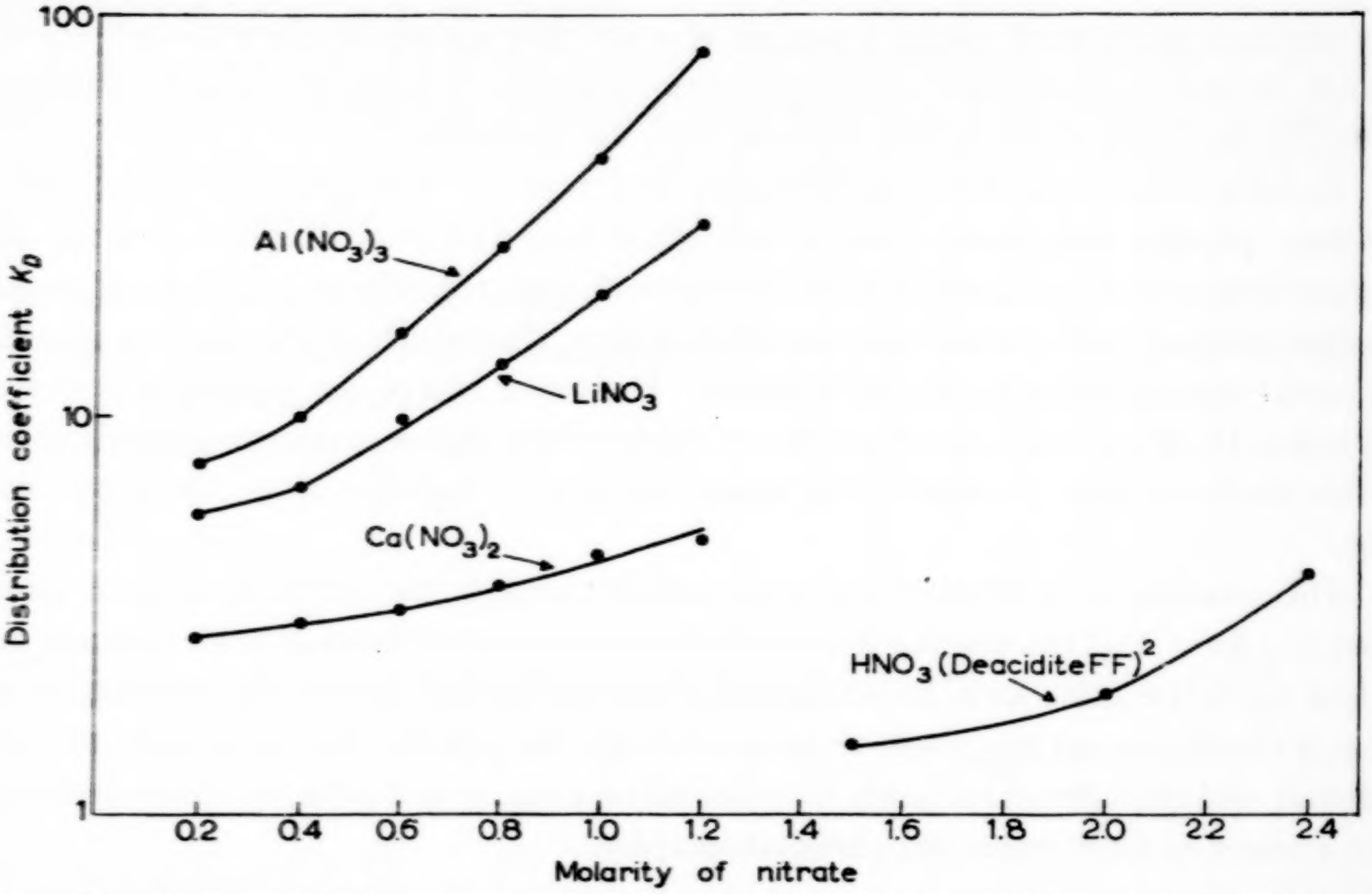

Fig. 1. Distribution coefficient of thorium as a function of concentration of various inorganic nitrate solutions.

The author wishes to thank the South African Atomic Energy Board for the award of a Senior Bursary.

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Received April 5th, 1961

Filtration and quantitative transfer of lambda amounts of liquids

The common problem of taking a sample of a few micrograms in a few microliters of a solvent, in order to transfer it to a paper chromatogram, is fairly frequently complicated by the fact that some of the material may be insoluble.

In such cases, quantitative filtration and transfer was achieved as follows: a capillary pipette was made from a soft glass tube (internal diameter 6 mm) and plugged firmly with a 3–4 mm wad of cotton-wool, with the help of a capillary glass rod. Another plug of cotton-wool was inserted close to the mouth of the pipette, in order to protect the operator in case of breakage. The lower end of the pipette was cut off just below the plug, and a small mark was made with a glass-cutter immediately above it; this makes it easy to remove the tip of the pipette together with the cotton-wool plug.

The solution to be filtered was then sucked through the cotton-wool plug, which acted as a filter, and the residue was washed with a few additional μl of the solvent and sucked up in the same way. This additional solvent served also for the washing of the filter. A small amount of air was then sucked into the pipette, the tip broken, the plug removed and the filtered contents transferred to a micro-test tube, or spotted directly onto a sheet of filter paper for chromatography.

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Received April 20th, 1961

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[*] The author received a grant from the National Research Council of Brazil.

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The preparation of lambda amounts of serum for paper electrophoresis

The study of serum proteins by means of paper electrophoresis when only very small amounts of blood are available presents a rather difficult problem. This occurs quite frequently when small animals, such as mice, are being used.

In the case of mice we have perfected the following technique: a sample of blood of about 100 μl was collected from the orbital vein by means of a capillary tube. It was then brought to the middle of the tube, by suction or just by tipping the tube, and the narrower end was sealed in a micro-bunsen burner. The tube was then cut at the level of the sample, wrapped in cotton-wool placed in a 15 ml centrifuge tube and centrifuged at 3,500 r.p.m. for 15 min.

This separates the clot at the bottom of the capillary tube, from which it can be easily removed by cutting the tube at the level of the clot. The serum so obtained can be easily transferred to a capillary pipette for measurement by touching the freshly cut end of the tube with the tip of the capillary pipette.

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BOOK REVIEWS

V. Congresso Nucleare, published in two volumes by the Rassegna Internazionale Elettronica Nucleare e Teleradiocinematografica, Via della Scrofa 14, Roma, 914 pages.

The two volumes containing the various symposia of the Fifth Nuclear Congress (June, 1960) have just appeared. The symposium on the preparation of radioisotopes for medical and industrial use (246 pages of Vol. 2), with contributions from Saclay, Oak Ridge, and other institutes throughout the world including Russia, contains much of interest to workers in the field of chromatography, such as descriptions of an ^{132}I generator and a ^{90}Y generator.

This is followed by a special session containing also those papers that were not grouped into special symposia. It includes a paper by SAUVAGNAC AND ROSA on the separation of K–Rb–Cs from fission products, one by BERTOLACCINI AND BERTOLACCINI on the use of organic substances as moderators and refrigerants in nuclear reactors, one by LO MORO, PUCINI AND RIGALI on the coprecipitation of radioelements with organic reagents and one by BECKMANN AND LEDERER on the adsorption chromatography of polonium on cellulose.

The volumes are attractively prepared and all papers are in English, French or Italian.

Praktikum der Papierchromatographie, Anleitungen zu Übungen in der papierchromatographischen Untersuchung pflanzlicher Inhaltsstoffe, by H. F. LINSKENS AND LUISE STANGE, Springer-Verlag, Berlin, 1961, 51 pages, 27 figures, price, with spiral binding, D.M. 9.80.

The authors of this book describe twelve exercises which are already being employed by them in a course of practical work in botany. After two preliminary exercises including the observation of a separation of dyestuffs, the effect of temperature and of the shape of the paper, the chromatography of amino acids, sugars, nucleic acids and their constituents, auxins, depsides, leaf pigments, anthocyanins and anthoxanthins, antibiotics and alkaloids is dealt with in such a way that each exercise introduces not only new substances but also new chromatographic and quantitative techniques. The final exercise consists in work with ^{14}C and its detection by radioautography and counting techniques.

In all the exercises suggestions for tabulation of the results as well as a number of questions are given to the students. The whole is carefully thought out and excellently planned. With minor modifications it may be used in practical biochemistry as well as in botany. The only drawback for some institutes may be that much of the work requires considerable equipment and material.

Announcement

UNSATURATED FATTY ACIDS TO BE DISTRIBUTED BY NATIONAL INSTITUTES OF HEALTH LIPID PROGRAM

A cooperative program sponsored by the Division of Research Grants and the National Heart Institute, National Institutes of Health, will soon distribute ampoules containing 100 mg of each of 8 unsaturated fatty acid methyl esters. Available in 99% purity will be esters of docosa-13-enoate (erucate), eicosa-11-enoate, octa-12-enoate, and octa-11-enoate; samples of 95% or greater purity will include eicosa-5,8,11,14,17-pentaenoate, docosa-4,7,10,13,16,19-hexaenoate, tetracosa-15-enoate (nervonate), and octadeca-6-enoate (petroselenate).

Qualified investigators desiring sets of these samples should describe briefly the research program for which they are to be used. The Committee advising this activity will attempt to fill all deserving requests, although aiming also to assure broad distribution to areas where they may be needed. Letters should be addressed in duplicate to Dr. WILLIAM H. GOLDWATER, Division of Research Grants, National Institutes of Health, Bethesda 14, Md., U.S.A.

A LUMPED-FILM MODEL FOR GAS–LIQUID PARTITION CHROMATOGRAPHY

PART II. EXPERIMENTAL EVALUATION OF ANALYTICAL SOLUTIONS

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(Received December 21st, 1960)

In Part I (*cf.* FUNK AND HOUGHTON[1]) the differential equations governing non-ideal gas–liquid partition chromatography (G.L.C.) have been derived and simplified by introducing the concept of a lumped liquid film in which the lumping point is the midpoint of the liquid film. The resulting equations were solved by finite difference methods using an IBM-704 digital computer so that the program could be used to simulate the behavior of a typical column under various operating conditions. However, if certain simplifying assumptions are made, it is possible to obtain certain analytical solutions to the differential equations of G.L.C. that are particularly useful for determining the effect of operating variables on the elution time, but are too complex to use to evaluate changes in shape of the elution curve (*cf.* FUNK AND HOUGHTON[2]).

If the assumptions of no pressure drop ($\partial P/\partial z = 0$) and linear solubility ($K_2 = 0$) are made in equations (1), (10), (12) and (14) of Part I[1], the following partial differential equations result:

$$\frac{\partial X}{\partial t} = E\frac{\partial^2 X}{\partial z^2} - u\frac{\partial X}{\partial z} - \frac{k_G RTA}{\varepsilon}(X - X_i) \tag{1}$$

$$c_i = K_1 P X_i \tag{2}$$

$$\frac{\partial c_m}{\partial t} = \frac{D_L}{\Delta y^2}(c_i - c_m) = \frac{k_G P}{\Delta y}(X - X_i) \tag{3}$$

These equations still allow for the presence of a finite diffusion rate and longitudinal dispersion. c_m is the concentration at the midpoint in the liquid film (*i.e.*, $c_m(z,h/2,t)$). It will be shown later that the lumping point can be chosen anywhere in the liquid film, so that $\Delta y = mh$ where m is an empirical factor measuring the average fractional penetration of the solute into the liquid film.

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ANALYTICAL SOLUTIONS FOR THE LUMPED-FILM MODEL

Two analytical solutions have been obtained (*cf.* FUNK AND HOUGHTON[2]) to eqns. (1), (2) and (3) by treating G.L.C. as a boundary value problem with time varying boundary conditions as shown in Fig. 1. A pulse of solute of mole fraction X_0 and duration b is introduced into the carrier gas stream at the column inlet. The initial conditions are therefore all zero, so that:

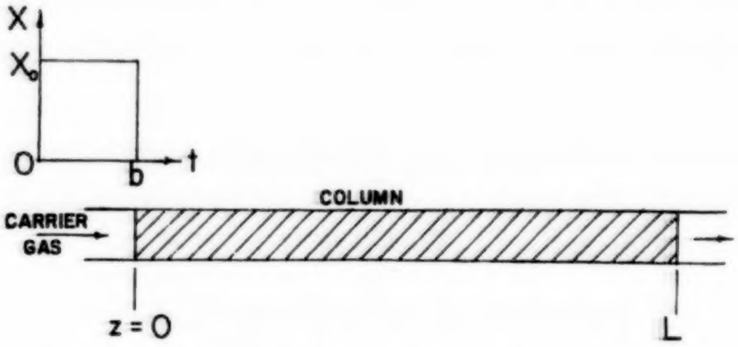

Fig. 1. The boundary value problem of G.L.C.

$$\begin{aligned} X(z,0) &= X_i(z,0) = 0 \\ c_m(z,0) &= c_i(z,0) = 0 \end{aligned} \tag{4}$$

and the time-varying boundary conditions are:

$$X(0,t) = \begin{cases} 0, & t < 0 \\ X_0, & b > t > 0 \\ 0, & t > b \end{cases} \tag{5}$$

If $E = 0$ in eqn. (1), the boundary value problem of Fig. 1 reduces to the initial value problem discussed in Part I with b equal to $L_1/u\,\varepsilon$ (*cf.* FUNK AND HOUGHTON[1], Fig. 2).

Subject to the boundary conditions of Fig. 1, two analytical solutions may be obtained to eqns. (1), (2) and (3) with and without longitudinal dispersion.

1. *Analytical solution for constant pressure, linear solubility and longitudinal dispersion* ($E \neq 0$)

The analytical solution for this case can be obtained by using a method similar to that outlined by LAPIDUS AND AMUNDSON[3]. However, the result summarized below distinguishes between rate processes taking place in both the gas and liquid phases and introduces physically measurable quantities that are characteristic of each process:

$$\begin{aligned} X(L,t) = \int_0^t \Bigg(X(0,t-\tau) \Bigg\{ \mathrm{e}^{\left(\frac{uL}{2E} - \gamma_1\tau\right)} \Bigg[\frac{L}{2\sqrt{\pi E \tau^3}}\, \mathrm{e}^{\left(\frac{-L^2}{4E\tau} - \gamma_3\tau\right)} - \\ - \frac{L\sqrt{\gamma_2 P}}{2\sqrt{\pi E}} \int_0^\tau \frac{1}{\lambda\sqrt{\tau-\lambda}}\, \mathrm{e}^{\left(\frac{-L^2}{4E} - \gamma_3\lambda\right)} J_1\left(\sqrt{\gamma_2 P \lambda\,(\tau-\lambda)}\,\right) \mathrm{d}\lambda \Bigg] \Bigg\} \Bigg) \mathrm{d}\tau \end{aligned} \tag{6}$$

where:

$$\gamma_1 = \frac{D_L}{\Delta y^2}\left[\frac{k_G}{\dfrac{D_L K_1}{\Delta y} + k_G}\right] \tag{7}$$

$$\gamma_2 = K_1\gamma_1 \tag{8}$$

$$\gamma_3 = -\gamma_1 + \frac{RTA\Delta y}{\varepsilon}\gamma_2 + \frac{u^2}{4E} \tag{9}$$

In eqn. (6) J_1 is a Bessel function of the first order and $X_0\,(0,t-\tau)$ is the boundary condition at the entrance to the column given by eqn. (5). The complexity of eqn. (6) makes it difficult to obtain a clear relationship between the retention time or the shape of the elution curve and the physical properties of the system. In this respect the numerical solution of Part I is simpler to use and in addition accounts for the effects of nonlinear solubility and pressure drop. Furthermore, by the use of the computer program in Part I, it has been demonstrated that although the longitudinal dispersion coefficient affects the shape of the elution curve, it does not affect the retention time. Therefore, by neglecting the effects of longitudinal dispersion, it is possible to obtain a simple relationship between the elution time and the physical variables in G.L.C., so that this case will be considered next.

2. *Analytical solution for constant pressure, linear solubility and no longitudinal dispersion* ($E = 0$)

The differential equations describing G.L.C. with no longitudinal dispersion are eqns. (1), (2) and (3) with $E = 0$. Although the final result for this case has already been quoted by FUNK AND HOUGHTON[2], the details have not previously been given.

Equations (1), (2) and (3) can be combined and written as follows:

$$\frac{\partial X}{\partial t} = -u\frac{\partial X}{\partial z} - \gamma_4 X + \gamma_5 c_m \tag{10}$$

$$\frac{\partial c_m}{\partial t} = \gamma_6 X - \gamma_1 c_m \tag{11}$$

where:

$$\gamma_4 = \frac{RTA\Delta y}{\varepsilon}\gamma_2 = \frac{K_1 RTA\Delta y}{\varepsilon}\gamma_1 \tag{12}$$

$$\gamma_5 = \gamma_4 / K_1 P \tag{13}$$

$$\gamma_6 = \gamma_2 P \tag{14}$$

Taking the Laplace transforms of eqns. (10) and (11) and combining the results by noting that $\gamma_1\,\gamma_4 = \gamma_5\,\gamma_6$, we obtain:

$$\frac{x(L,s)}{x(0,s)} = \exp\left[-\frac{L}{u}\,s\left(\frac{s+\gamma_1+\gamma_4}{s+\gamma_1}\right)\right] \tag{15}$$

The ratio $x(L,s)/x(0,s)$ will be referred to as the column transfer function. If a pulse of duration b and height X_0 is used for the input, $x(0,s)$ (*cf.* Fig. 1 and eqns. (4) and (5)), then the inverse transform can be obtained by convolution utilizing the two theorems below:

$$\mathscr{L}^{-1}\left[\frac{\mathrm{d}}{\mathrm{d}\gamma_2}g(s)\right] = \frac{\mathrm{d}}{\mathrm{d}\gamma_2}G(t) \tag{16}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\left[t^p I_p(at)\right] = at^p I_{p-1}(at) \tag{17}$$

The inverse transform is then:

$$\frac{X(L,t)}{X_0} = \begin{cases} 0, \quad 0 < t < L/u \\ \displaystyle\int_{t-b}^{t} \mathrm{e}^{-\gamma_1\left(\tau - \frac{L}{u}\right) - \frac{L}{u}\gamma_2} \sqrt{\frac{\gamma_1\gamma_2 L/u}{\tau - \frac{L}{u}}}\, I_1\left[2\sqrt{\gamma_1\gamma_2\frac{L}{u}\left(\tau - \frac{L}{u}\right)}\right]\mathrm{d}\tau, \quad t > L/u \end{cases} \tag{18}$$

If it is first assumed that the duration of the pulse is short ($b \ll L/u$), secondly that the modified Bessel function, I_1, can be approximated by its asymptotic expansion and thirdly that $\lim\limits_{b\to 0}\int_{t-b}^{t} f(\tau)\mathrm{d}\tau = bf(\tau)$, then the retention time can be obtained by determining the time at which the resulting function maximizes (*cf.* FUNK AND HOUGHTON[2]). The result of applying this procedure is as follows:

$$t_R = \frac{L}{u}\left(1 + \frac{K_1 RTA\Delta yF}{\varepsilon}\right) \tag{19}$$

where:

$$F = \frac{1}{2} - \frac{3u}{4\gamma_2 L} + \frac{1}{2}\sqrt{1 - \frac{3u}{\gamma_2 L}} \tag{20}$$

It is the factor F in the above equation that allows for the effect of a finite mass transfer rate on the retention time. Under the normal operating conditions of G.L.C. the liquid film is thin and the column is long, so that F is very close to unity. Further, since the apparent retention time, t'_R, is t_R minus the transport time without chromatography, L/u, we obtain:

$$t'_R = \frac{LK_1 RTA\Delta y}{u\varepsilon} \tag{21}$$

If the lumping point is taken as the midpoint of the liquid film, then $\Delta y = h/2$ and the volume of stationary liquid per unit volume of column, V, is equal to Ah, so that:

$$t'_R = \frac{1}{2}\frac{LK_1 RTV}{u\varepsilon} \tag{22}$$

However, it is possible to choose the lumping point anywhere in the liquid film, so that this case will now be considered separately as the penetration model.

PENETRATION MODEL

Referring to Part I (Fig. 1 and eqns. (11) to (15)), it may easily be seen that the lumping points varies with time as the solute passes into the liquid film and then out again. It is clear that the lumping point for a single section approximation is defined in such a way that the concentration gradient on one side is large and on the other side, small. Since the position of this lumping point varies only with time, it is possible to define a time-average penetration depth, mh, such that $\Delta y = mh$. If the lumping point is arbitrarily taken as the midpoint of the liquid film $m = 1/2$, which accounts for the factor of $1/2$ in eqn. (22), but assuming an average fractional penetration depth of m, eqn. (22) becomes:

$$t'_R = m \frac{LK_1RTV}{u\,\varepsilon} \tag{23}$$

where m may vary between zero and unity.

Eqn. (23) can be put in a more usable form by combining Raoult's law with the Clausius-Clapeyron equation for the vapor pressure, p^0, to obtain K_1 as a function of temperature. The useful forms of eqn. (23) may be summarized as follows:

$$t'_R = m \frac{L\rho_M RTV}{p^0 u\,\varepsilon} = m \frac{L\rho_M RTV}{Bu\,\varepsilon} \exp(\Delta H/RT) \tag{24}$$

B is the pre-exponential constant in the Clausius-Clapeyron equation and ρ_M is the molal density of the stationary liquid phase.

COMPARISON OF THE LUMPED-FILM MODEL AND THE EQUILIBRIUM MODEL

LAPIDUS AND AMUNDSON[3] have obtained an analytical solution for the case where equilibrium between the liquid and vapor phases is established instantaneously at any point in the column. Their result can be obtained by making D_L and k_G infinite and setting $\Delta y = h$ in eqn. (15). The Laplace transform for this model can be obtained by letting $\gamma_1 \to \infty$ in eqn. (15) and using L'Hospital's rule on the exponent of the exponential. The column transfer function then becomes:

$$\frac{x(L,s)}{x(0,s)} = \exp\left[-\frac{L}{u}\,s\left(1 + \frac{K_1RTAh}{\varepsilon}\right)\right] \tag{25}$$

Inspection of transform (25) shows that the equilibrium model simply leads to a pure time delay of $LK_1RTAh/u\varepsilon$ beyond the normal transport time of L/u, so that remembering that $V = Ah$, we obtain:

$$t'_R = \frac{LK_1RTV}{u\,\varepsilon} \tag{26}$$

Furthermore, it is clear from eqn. (25) that the equilibrium model leads to no change in either the size or the shape of the pulse. However, the transform (15), obtained by using the lumped-film model, leads to eqn. (18) which shows that a finite rate of chromatography, introduced by the coefficients D_L and k_G, not only produces a delay beyond the transport time, but also produces a change in the shape of the elution curve. The effect of k_G on the elution curve and retention time has already been illustrated in Part I by the use of the computer program.

Comparison of eqns. (22), (23) and (26) shows that the average fractional depth of penetration, m, for the simple lumped-film model is 1/2, while that for the equilibrium model is 1.0. Experiments, that will now be discussed, have shown that in actual practice, m has values in the range 0.6 – 0.9, depending largely upon the film thickness and the slug length, and that at constant film thickness the assumption of a constant value for m in eqn. (24) is remarkably good.

EXPERIMENTAL

In order to evaluate the application of eqns. (23) and (24) to elution times actually observed in G.L.C., an apparatus was constructed that conformed as closely as possible to the boundary conditions of Fig. 1. The solute used was isobutylene (Matheson CP) in a carrier stream of helium. The stationary phase was dinonyl phthalate (National Research Corporation, specially refined Narcoil-40) uniformly coated on to 30–60 mesh firebrick (Burrell Corporation Kromat-FB).

The slug of pure isobutylene ($X_0 = 1$) was confined in a slug chamber between two stopcocks lubricated with glycerine. Two slug chambers were available, one holding 3.0 cm^3 and the other 6.7 cm^3 of gas. They could be filled through capillary side arms connected to the isobutylene supply. Arrangements were made to divert the helium stream to the top of the chromatographic column while the slug chamber was being filled. To introduce the slug into the helium stream, it was only necessary to open the two stopcocks and divert the carrier gas from the top of the column back through the slug chamber. The slug entered the packed bed almost as soon as it issued from the slug chamber. The chromatographic column itself and the slug chambers were constructed from 7 mm i.d. Pyrex glass tubing and the column was packed to a length of about 100 cm. The detector was a thermal conductivity gauge constructed from the tungsten filament of a Westinghouse No. 1819 miniature light bulb and was placed directly in the gas stream only 3.5 cm from the end of the packed section. Another similar filament was placed 100 cm downstream in the same tube to compensate for flow variations. The two filaments formed two arms of a chopper-stabilized Wheatstone bridge, the unbalance voltage being recorded on a Leeds and Northrup Azar recorder with a time constant of 1 sec and a chart speed of 3 in. per minute. Since the detectors were bare filaments placed directly in the gas stream, their time constants were very low, so that no errors in the elution time could be attributed to measurement lags. The slug chamber, the chromatographic column and the detectors were all surrounded by a water jacket provided with water circulating from a thermostated bath. In this way the column temperature could be maintained at any temperature in the range 20–80° to within $\pm$ 0.05°. Carrier gas flow rates in the range 7–70 cm³/min could be accurately measured within $\pm$ 0.5% by means of a soap bubble flow meter. The pressure drop across the columm was less than 7.5 cm Hg for all the flow rates used. The volumetric flow rates, Q, for use in the equations were calculated at the average column pressure.

To make a measurement, the elution time of an isobutylene slug was first measured in the wet column; the transport time without chromatography was then measured under the same conditions using a nitrogen slug. However, by separate experiments conducted on dry firebrick, it was found that there was approximately a 10% difference in the transport times between nitrogen and isobutylene, the latter taking longer to appear. Typical results for dry firebrick are shown in Fig. 2—it is interesting to note that temperature has no detectable effect on the transport time of either isobutylene or nitrogen in the range 31–60°, indicating perhaps that adsorption is not the cause of the delay in the case of isobutylene. The reason for the delay is believed to be that isobutylene diffuses into and out of the pores in firebrick more slowly than nitrogen. By assuming that the pores in the wet firebrick were not filled with partitioning liquid, it was possible to make a correction for the difference in transport times of nitrogen and isobutylene, using the data from the dry column. The apparent retention time, t'_R, could then be computed by subtracting the corrected transport time, L/u, from the total elution time, t_R.

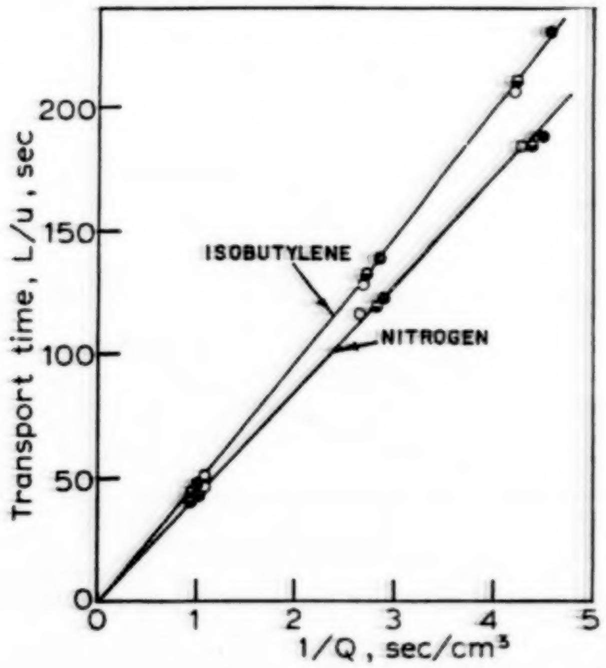

Fig. 2. Transport times through dry firebrick. $V_g = 6.7$ cm³. ● 31.0°; ◒ 45.0°; ○ 60.0°.

RESULTS AND DISCUSSION

Equations (23) and (24) may be applied to the experimental data by substituting $Q = ua\varepsilon$ and $V_p = LaV$, where a is the cross-sectional area of the column, Q is the volumetric flow rate of carrier gas and V_p is the total volume of partitioning liquid present in the chromatographic column; the result is:

$$t'_R = \frac{mK_1RTV_p}{Q} = \frac{m\rho_M RTV_p}{p^0Q} = \frac{m\rho_M RTV_p}{BQ} \exp\,(\Delta H/RT) \qquad (27)$$

According to eqn. (27), a plot of the apparent retention time t'_R versus $1/Q$ should be a straight line; Fig. 3 shows typical plots for various amounts of partitioning liquid at constant temperature. When the points of Fig. 3 were plotted on a larger scale, it was found that, although the lines were straight, they extrapolated backwards to give a small negative intercept on the t'_R axis when $1/Q = 0$. The cause of this effect is not certain, but it could be due to unsteady state flow effects during the introduction of the slug, or perhaps to the non-uniform velocity distribution at the unpacked entrance to the column, or even to the effects of the finite rate of mass transfer on fluid flow in the region of the slug. It is interesting to point out that a finite mass transfer rate normal to a flowing fluid would be equivalent to increasing the effective viscosity of the carrier gas. However, the intercepts were small compared with the elution times in the present experiments.

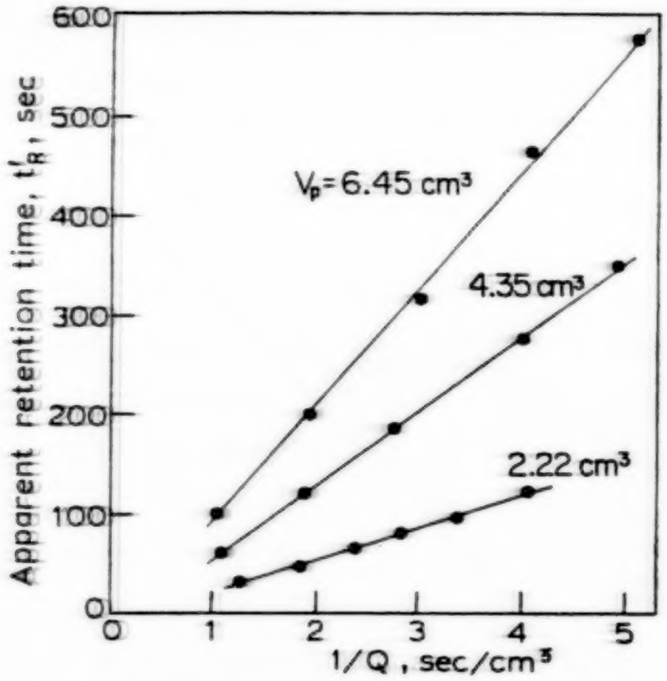

Fig. 3. Effect of amount of dinonyl phthalate on the elution of isobutylene at 31.0°. V_s = 6.7 cm³.

Fig. 4 has plots of t'_R *versus* $1/Q$ for three different temperatures, showing that as the temperature is reduced, the elution time increases, as would be expected from eqn. (27). If the carrier velocity is constant, then according to eqn. (27) a plot of $\log\,(t'_R/T)$ *versus* $1/T$ should be a straight line of slope $\Delta H/R$, where ΔH is the latent heat of vaporization of isobutylene. Typical plots are shown in Fig. 5 for three different carrier gas velocities. The average of the slopes of the three lines gives $\Delta H = 5.30 \pm 0.05$ kcal/g-mole, compared with a value of 5.4 ± 0.2 kcal/g-mole calculated from the isobutylene vapor pressure data of COFFIN AND MAASS[4].

To determine the fractional penetration depth, m, the data of Fig. 4 were replotted as t'_R/K_1RTV_p *versus* $1/Q$ so that according to eqn. (27) the slope should be m.

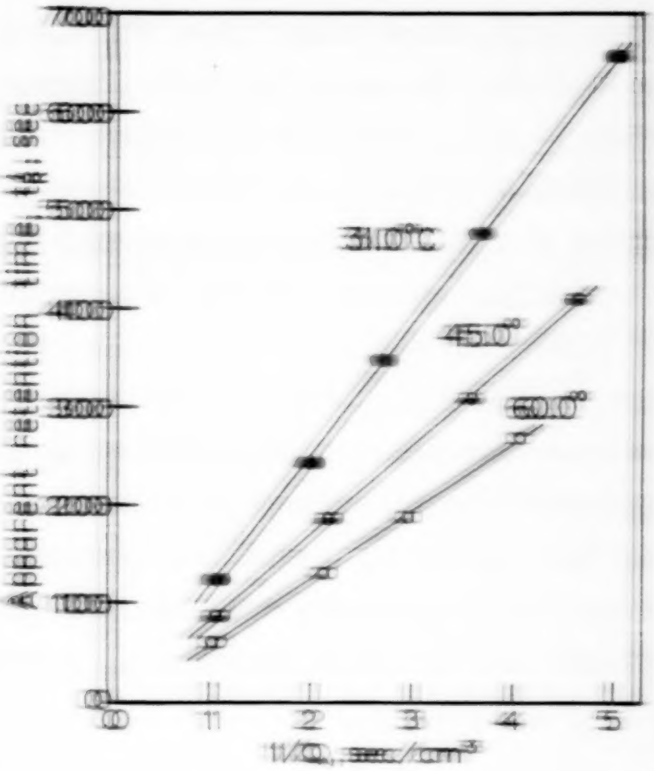

Fig. 4. Effect of temperature on the elution of isobutylene slugs using dinonyl phthalate on firebrick. V_s = 3.0 cm³; V_p = 6.45 cm³.

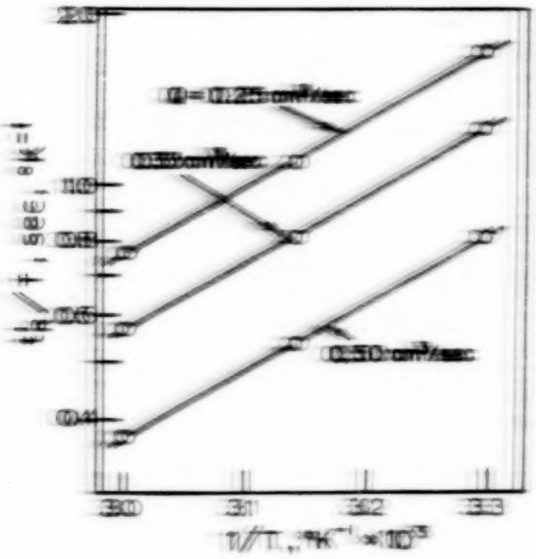

Fig. 5. Plot of log (t'_R/T) *versus* 1/T for the isobutylene–dinonyl phthalate system. V_s = 3.0 cm³; V_p = 6.45 cm³.

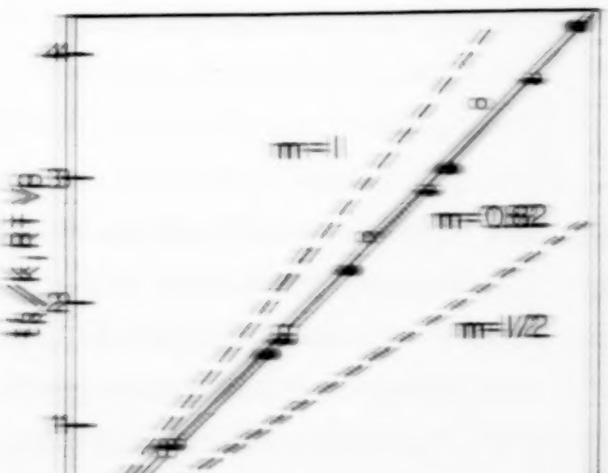

Fig. 6. Evaluation of the time-average penetration depth, *m*. V_s = 3.0 cm³; V_p = 6.45 cm³. ● 31.0°; ◐ 45.0°; ○ 60.0°.

Values of K_1 were taken from the data of HOUGHTON, KESTEN, FUNK AND COULL[5]. The best line through the data gives a value of $m = 0.82$ and correlates the data within $\pm 2\%$, with the exception of one point. The lines for $m = 1/2$ and $m = 1$ (equilibrium theory) are also shown in Fig. 6. It is evident that data on the same column at various temperatures and for a fixed slug length are very well correlated by a constant average depth of penetration.

Table I shows the effect of slug length, amount of partitioning liquid and temperature on the time-average fractional depth of penetration, m. The fact that m increases as the amount of partitioning liquid increases indicates that the effect of the impermeable solid support is significant at small film thicknesses. However, it is also clear that m is not a sensitive parameter, since its values are limited to the range 0.6–0.9 for the present experiments, and in fact an overall average value of 0.72 will correlate all the data in Figs. 2, 3 and 4 and Table I with an accuracy of better than $\pm 25\%$.

TABLE I

TIME AVERAGE PENETRATION DEPTH, m, FOR THE ISOBUTYLENE–DINONYL PHTHALATE SYSTEM ON FIREBRICK

Temperature °C	*Slug volume* V_S, *cm*^{3}	*Wt. % dinonyl phthalate*	*Solubility* $K_1 \times 10^6$ *g-mole/cm*^{3}*.atm.*	*Volume dinonyl phthalate* V_p, *cm*^{3}	m
31.0	6.7	10.1	9.64	2.22	0.60
31.0	6.7	17.9	9.64	4.35	0.70
31.0	6.7	24.2	9.64	6.45	0.74
31.0	3.0	24.2	9.64	6.45	0.81
45.0	3.0	24.2	6.34	6.45	0.81
60.0	3.0	24.2	4.17	6.45	0.84

NOTATION

a = cross-sectional area of chromatographic column, cm^2
A = mass transfer area per unit volume of empty column, cm^2/cm^3
b = duration of solute pulse, sec
B = pre-exponential constant in Clausius-Clapeyron equation, atm
c = solute concentration in liquid phase, g-mole/cm^3
c_i = interfacial solute concentration, g-mole/cm^3
c_m = solute concentration at lumping point in liquid film, g-mole/cm^3
D_L = molecular diffusivity in liquid phase, cm^2/sec
E = longitudinal dispersion coefficient cm^2/sec
F = dimensionless factor given by eqn. (20)
h = liquid film thickness, cm
ΔH = latent heat of vaporization of solute, cal/g-mole
I_p = modified Bessel function of order p
J_1 = Bessel function of order unity

k_G = gas film coefficient, g-mole/sec.cm².atm
K_1, K_2 = constants in nonlinear solubility isotherm
L, L_1 = length, cm
$\mathscr{L}$ = Laplace transform
m = time-average penetration depth, dimensionless
P = column pressure, atm
p^0 = vapor pressure of solute gas, atm
Q = volumetric gas flow rate, cm³/sec
R = Universal Gas Constant, 82.06 atm. cm³/g-mole. °K
s = Laplace parameter, complex variable
t = time, sec
t_R = retention time, sec
t'_R = apparent retention time, sec
T = absolute temperature, °K
u = axial gas velocity through voids, cm/sec
W = volume of partitioning liquid per unit volume of empty column, dimensionless
W_p = total volume of partitioning liquid in chromatographic column, cm³
W_s = volume of slug, cm³
X = mole fraction of solute in vapor phase
X_i = mole fraction of solute at gas–liquid interface
X_0 = initial mole fraction of solute in slug
Δy = incremental distance in liquid film, cm
z = axial distance along column, cm
$\gamma_1, \gamma_2, \gamma_3, \gamma_4, \gamma_5, \gamma_6$ = parameters defined in text
ε = void fraction, dimensionless
λ = variable of integration
ρ_M = molal density of liquid phase, g-mole/cm³
τ = variable of integration

ACKNOWLEDGEMENT

One of us (J.E.F.) wishes to express his gratitude to the Westinghouse Corporation for a graduate fellowship.

SUMMARY

The differential equations governing gas–liquid partition chromatography have been simplified by using the assumptions of linear solubility and no pressure drop and introducing the concept of a lumped liquid film. Analytical solutions of the equations have been obtained for the cases with and without longitudinal dispersion. The solutions show that a finite rate of mass transfer into the liquid phase can affect both the shape of the elution curve and the retention time. A simple equation has been obtained relating the apparent retention time to the column variables and the depth of penetration into the liquid film. Experimental data on the elution of a pure iso-

butylene slug with helium using a stationary phase of dinonyl phthalate on firebrick has shown the average fractional depth of penetration to be in the range 0.6–0.9 under normal operating conditions. It was found that the penetration model correlates the retention time at various temperatures remarkably well, provided the slug size and the amount of stationary phase are held constant.

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GAS-LIQUID CHROMATOGRAPHY IN QUALITATIVE ANALYSIS

PART II. THE REPRODUCIBILITY OF RETENTION DATA IN R_9 UNITS

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(Received December 23rd, 1960)

INTRODUCTION

The presentation of retention data in absolute units (specific retention volumes or partition coefficients), although conceptually simple, is beset with practical difficulties if reasonable accuracy is to be obtained[1]. For analytical purposes, retention ratios are generally used. However, owing to the wide range of column conditions and solutes concerned no single conventional standard material suffices. A wide range of arbitrary standards are therefore to be found in the literature.

This situation is unsatisfactory from a number of aspects. Thus, filing and tabulation of data become cumbersome, comparison of data using different standards is difficult and thermodynamic relationships are indeterminate.

A note has been published[2] by one of the present authors which shows that retention ratios relative to *n*-nonane can be obtained by extrapolation or interpolation of the close linear relation between log (retention) and carbon number in the homologous *n*-paraffin series. This is referred to as the "log plot" in this paper. The reasons for the particular choice of *n*-nonane as standard are given later.

The precision of the method clearly depends on the precision with which the retentions of the *n*-paraffins, or any other internal standards, can be expressed relative to the retention of *n*-nonane which in turn depends on the accuracy of the interpolation or extrapolation of the linear "log plot". This is discussed in some detail in the theoretical section.

EXPERIMENTAL

Apparatus

Only conventional and simple gas chromatography equipment was used throughout this investigation. Columns were 5 feet × 4 mm in pyrex glass using a modified flame ionisation detector[3]. The carrier gas was a 3:1 (by volume) mixture of hydrogen and nitrogen. The carrier gas flow rate and column inlet pressure were controlled only by an OR12 pressure regulator (British Oxygen Gases Ltd.). The column temperature was regulated by a vapour jacket consisting of boiling methanol (64.7°), water (100.0°) or *n*-pentanol (138.0°). These boiling points were subject to some variation due to changes in barometric pressure which ranged from 740 to 765 torr. The stationary phases were supported on acid-washed (1% phosphoric acid) 60–72 mesh celite (J.J.'s, Ewell, Surrey, England) and in certain cases on 60–72 mesh C 22 firebrick (J.J.'s).

In this investigation the only stationary phases used were dinonyl phthalate (BDH for chromatography) and squalane (BDH for chromatography). In our experience, polyethylene glycol and silicone columns, although otherwise useful, change their character on usage even at quite moderate temperatures. Samples (approximately 10 γ) for analysis were introduced by means of stainless steel capillary pipettes[4]. Columns were operated at or near optimum carrier gas flow rate (approx. 50 c.c./min, measured at room temperature and at column outlet pressure) and had efficiencies between 400 and 500 theoretical plates per foot of column.

Materials

Solutes were selected with a view to having as wide a range of solvent interactions as possible. Thus in respect of dipole moments these ranged from zero for the *n*-paraffins to about 4.0 Debye units for aromatic nitro compounds. Group polarisabilities ranged from 1.68 for the C—H bond of *n*-paraffins to 8.1 for the S—S bond in disulphides (polarisability expressed as contributions to the Lorentz molar refraction). Hydrogen bond acceptor and donor properties ranged from zero in the *n*-paraffins to the strong tendency in both respects for alcohols.

The solutes studied were all commercially available and were either predominantly pure (checked by infra-red analysis and gas–liquid chromatography where appropriate) or had a component which was clearly identifiable (*e.g.* from a homologous series gas–liquid chromatography plot).

Calculation procedure for R_{x9} values

A convenient calibration series of *n*-paraffins was injected into the column and a chromatogram obtained. Since the overall reproducibility was being investigated this was repeated for each R_{x9} value determined. In normal practice this calibration chromatogram needs only to be repeated at infrequent intervals to check any change of column characteristics with usage.

Retention *distances* on the chart were then measured from the "column dead volume point" to the peak maxima.

With detectors insensitive to air no "air peak" is observed and this presents some difficulty. This was obviated by the following procedure. A calculated value for the column interstitial volume was first taken, converted into chart distance, and subtracted from retention distances measured from the point of injection. These adjusted retention distances were then plotted against carbon number (on 11½ in. × 16½ in. 2 or 3 cycle log-linear graph paper). If in fact the calculated interstitial volume was in error some curvature of the plot at low retention times was apparent. The further correction required could, however, easily be computed from the expression

$$\delta a = \frac{2.303 \sum [R(\delta \log R)^2]}{\sum [\delta \log R]}$$

where R = retention distance from assumed dead volume point on chart

$\delta \log R = \log R_{\text{observed}} - \log R_{\text{extrapolated}}$

$R_{\text{extrapolated}}$ = value obtained from extrapolation of linear part of graph

δa = further correction to retention distance to be subtracted from the first set of adjusted retention distances.

Provided the first calculated interstitial volume of the column was not seriously in error the above correction gave very precise values for adjusted retention distances and on replotting the data no curvature was apparent.

From the corrected graph the retention distance for *n*-nonane was obtained by interpolation or extrapolation. Within the temperature range studied this could be done graphically with sufficient accuracy. At very much higher temperatures "least squares" methods *may* be required.

From the value for the adjusted retention distance of *n*-nonane obtained from the corrected graph, the relative retentions of the *n*-paraffins within the calibration with respect to *n*-nonane were calculated from the relation

$$R_{N_9} = R_N / R_9$$

where R_{N_9} = retention of paraffin relative to *n*-nonane

R_N = adjusted retention distance on chart of the paraffin

R_9 = adjusted retention distance on chart of *n*-nonane deduced from the graph.

On occasion the use of *n*-paraffins as internal standards is inconvenient. In this case a suitable internal standard was added to the calibration series and the retention of the proposed internal standard relative to *n*-nonane thus obtained. On very polar stationary phases the concentrations of *n*-paraffins in this calibration generally need to be small in order to obviate band broadening due to delayed volatilisation. With the high sensitivity detectors now available this presents little difficulty.

Having thus obtained the retentions of the *n*-paraffins or other standards relative to *n*-nonane one or more of these were used as conventional internal standards to measure the retentions of various solutes relative to *n*-nonane from the relation

$$R_{x_9} = R_{xN} \times R_{N_9}$$

where R_{x_9} = retention of material being measured relative to *n*-nonane

R_{xN} = retention of material being measured relative to the internal standard

R_{N_9} = retention of standard relative to *n*-nonane.

For reasons discussed in the theoretical section, wherever possible, two internal standards eluting either side of the material being measured, were used and the mean R_{x_9} value taken.

For calibration purposes only *n*-paraffins above *n*-butane should be used since on theoretical grounds the linearity of the "log plot" should deteriorate below *n*-butane. This is not generally of concern since these low molecular weight paraffins are more conveniently analysed by gas–solid chromatography.

Temperature cycling of columns

In order to simulate normal practice in analytical laboratories the columns were temperature cycled as follows: (1) Dinonyl phthalate columns, 65° (7 h), 100° (7 h), 65° (7 h), 100° (7 h) etc. (2) Squalane columns, 65° (7 h), 100° (7 h), 138° (7 h), 65° (7 h) etc.

In the lower temperature range it is possible that the reproducibility of retention data would be better than that observed if the column was not operated at higher temperatures.

THEORETICAL

The reproducibility of conventional retention ratios

The simplest case to consider is where the standard and the unknown are run on separate chromatograms. If it is assumed that the percentage standard deviation of a retention time is independent of retention time (this has experimental support provided rapidly eluted materials are excluded) then the theory of random error propagation predicts that

$$\varepsilon_{x/s} = (\varepsilon_x^2 + \varepsilon_s^2)^{\frac{1}{2}} = 2^{\frac{1}{2}}\varepsilon_0 \tag{1}$$

where $\varepsilon_{x/s}$ = % standard deviation in the retention ratio

$\varepsilon_x = \varepsilon_s = \varepsilon_0$ = % standard deviation in retention time.

In practice, however, the standard and the unknown are almost always run together on the same chromatogram (internal standard technique). The theoretical discussion of the standard deviation of the retention ratio under these conditions is complicated except for the unique cases (1) where the errors in operating conditions are subject to rapid random fluctuations or (2) where the operating conditions are subject to systematic linear drift. Under the latter conditions and taking the special case where errors are due to a linear drift in carrier gas flow rate then

$$\varepsilon_{x/s} = \frac{100a}{2F_0}\left|t_2 - t_1\right| \tag{2}$$

where $F = F_0 - at$ = carrier gas flow rate at time t

F_0 = flow rate at the time of elution of the "air peak"

t_1 = elution time of standard (measured from air peak)

t_2 = elution time of unknown (measured from air peak).

Clearly the error will be zero when $t_1 = t_2$, and hence the desirability of using an internal standard with a similar retention time to the unknown. Exact equality is not practicable since the peak must be resolved and an infinite range of standards would be required.

If two internal standards can be used which have a precise known relation to some primary standard then the percentage error of the mean retention ratio relative to this primary standard under conditions of linearly drifting carrier gas flow rate will be given by

$$\varepsilon_{x/p} = \frac{100a}{2F_0}\left|t_2 - \frac{(t_3 + t_1)}{2}\right| \tag{3}$$

where t_1 = retention time of first standard

t_2 = retention time of unknown

t_3 = retention time of second standard

and, $t_1 < t_2 < t_3$.

If therefore the retention of the unknown is the mean of the retentions of the two internal standards, errors due to a linearly drifting carrier gas flow rate will be eliminated. This is in principle a practical proposition since all peaks can be resolved under these conditions. Moreover although exact equality of t_2 and $(t_3 - t_1)/2$ can rarely be obtained, approximate equality pertains when the unknown is eluted between the two internal standards. This is a readily obtainable condition with the R_{x9} method using two convenient *n*-paraffins as internal standards.

Similar but more complex considerations apply to small linear drifts in other operating conditions (temperature, amount of stationary phase in column) provided the *character* of the stationary phase is not drifting. When the drift is no longer linear with time the errors can be greater than those suggested by eqns. (2) and (3).

The following general principles, however, still apply namely (1) Retention ratios measured on a single chromatogram should be more reproducible than those derived from separate chromatograms. (2) The accuracy with the former method should improve as the retentions of the internal standard and the unknown approach each other. (3) A method which involves the use of two internal standards more or less symmetrically disposed either side of the unknown should be more reproducible than methods using a single standard similarly disposed, provided the relations between the internal standards and the primary standard are precisely known.

The reproducibility of retention ratios in theoretical nonane units (R_{x9})

Since $R_{x9} = R_{xN} \times R_{N9}$, then by the law of random error propagation

$$\varepsilon_{x9}^2 = \varepsilon_{xN}^2 + \varepsilon_{N9}^2 \qquad (4)$$

This applies when the two determinations are on separate chromatograms. ε_{xN} is the % standard deviation for a conventional retention ratio. The critical factor therefore which will determine the % standard deviation of retention ratios in R_{x9} units is the quantity ε_{N9} which is the percentage standard deviation for the retention of the standard relative to *n*-nonane. This itself is a composite entity since

$$R_{N9} = R_N/R_9$$

where R_N = retention of standard

R_9 = retention of *n*-nonane.

Under the worst and never used conditions where these retentions are measured on separate chromatograms

$$\varepsilon_{N9}^2 = \varepsilon_N^2 + \varepsilon_9^2 \qquad (5)$$

Although these conditions never apply in practice it is nevertheless important to evaluate the probable value of ε_9, the % standard deviation for the retention of *n*-nonane using separate chromatograms throughout.

The value of R_{99} as stated previously is obtained by extrapolating the best straight line of the log R_N *vs.* carbon number plot of the *n*-paraffins. The probable error for R_{99} in such a calibration is given by

$$\varphi_{99}^2 = \frac{\sum (i - 9)^2}{v \sum i^2 - (\sum i)^2} \tag{6}$$

where φ_{99} = % probable error of R_{99}/% probable error of a single retention time
i = the carbon number of each paraffin in the calibration
v = the number of paraffins in the calibration.

This equation applies to the worst possible system of calibration where each paraffin is run on a separate chromatogram. Under normal calibration conditions of a single chromatogram for the whole series, φ_{99} may be expected to be much smaller.

The value of φ_{99} is clearly a function of the calibration range of *n*-paraffins. If all the *n*-paraffins with carbon numbers between m and n are used in the calibration eqn. (6) reduces to

$$\varphi_{99}^2 = \frac{2\{54(9 - n - m) + 2(n^2 + m^2 + mn) + n - m\}}{(n - m)(n - m + 1)(n - m + 2)} \tag{7}$$

or in more general terms

$$\varphi_{\mathcal{N}}^2 = \frac{2\{6\mathcal{N}(\mathcal{N} - n - m) + 2(n^2 + m^2 + mn) + n - m\}}{(n - m)(n - m + 1)(n - m + 2)} \tag{8}$$

where $\mathcal{N}$ is the carbon number of the *n*-paraffin primary standard.

By using eqn. (7) it is possible to evaluate the minimum value of n for each value of m which will make $\varphi_{99} \leqslant 1$.

The minimum calibration ranges shown in Table I have been selected on this basis.

In practice using a single chromatogram for the whole calibration these ranges are more than sufficiently wide and the accuracy correspondingly greater. By partially differentiating eqn. (8) with respect to $\mathcal{N}$, $\varphi_{\mathcal{N}}^2$ can be shown to have a minimum value when $\mathcal{N} = (n + m)/2$. That is to say the best primary standard from this point of view is one near the middle of the range of *n*-paraffins which are amenable to GLC.

Although *n*-nonane is somewhat below the middle of the range of *n*-paraffins which are amenable to GLC, primary standard paraffins with a higher carbon number would make calibration times at the lower end of the scale impossibly long. *n*-Nonane would therefore undoubtedly appear to be the best standard from a practical point of view.

The maximum practicable calibration range of *n*-paraffins is determined by the maximum convenient elution time for the last peak (R_n). Now owing to the linearity of the log plot

$$R_n = R_m \, 10^{b(n - m)} \tag{9}$$

where R_m = retention time of first peak (measured from air peak)
b = slope of log plot.

TABLE I

MINIMUM AND MAXIMUM [illegible] SELECTED FOR SMALLEST NUMBER OF [illegible]*

m	n	n max**	[illegible]	[illegible]	[illegible]	[illegible]
1	8		−[illegible]	[illegible]	11	[illegible] · $10^{[illegible]}$***
2	8		−[illegible]	7	11	[illegible] · $10^{[illegible]}$***
3	8		−[illegible]	[illegible]	11	[illegible] · $10^{[illegible]}$***
4	7	[illegible]	−29	[illegible]	5	470
5	9	[illegible]	[illegible]	5	11	[illegible]
6	9	111	[illegible]	4	11	[illegible]
7	9	122	[illegible]	3	11	14
8	9	144	[illegible]	2	11	5
9	10	[illegible]	[illegible]	2	11	4
10	15	[illegible]	[illegible]	[illegible]	11	[illegible]
11	18	211	[illegible]	[illegible]	11	97
12	20	225	[illegible]	[illegible]	11	[illegible]
13	22	[illegible]	[illegible]	10	11	[illegible]
14	24	[illegible]	[illegible]	11	11	[illegible]
15	26	333	[illegible]	[illegible]	11	[illegible]
16	28	337	[illegible]	[illegible]	11	[illegible]
17	30	445	[illegible]	[illegible]	11	25

* Columns 3, 4 and 7 in this Table [illegible] and refer to a 5% silicone 704 column of the type described under EXPERIMENTAL.

** n max is the highest value of n for the above column for which the time for a chromatogram does not exceed 8 h.

*** These values are impossibly [illegible].

The minimum practical value of $R_{\min}$ is that which can be accurately measured on the chart. With conventional potentiometric recorders having a maximum chart speed of 1 in. per min this minimum accurately measurable elution time is probably 1–2 min.

The factor b depends critically on the nature of the stationary phase and the temperature. Representative values for b are shown in Table II.

The maximum calibration ranges shown in Table I are for a 5% silicone MS704 column (4 mm × 5 feet) at temperatures appropriate to a retention time of about

TABLE II

EXPERIMENTAL VALUES OF b FOR [illegible] IN THE EQUATION $\log R = a - bN$

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
$10^3 \times 1/T$	3.413	[illegible]	[illegible]	[illegible]	[illegible]
PEG*	[illegible]	[illegible]	[illegible]	[illegible]	
DNP	[illegible]	[illegible]	[illegible]	[illegible]	
Sil 704*	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Squalane	[illegible]	[illegible]	[illegible]	[illegible]	

* These values are very approximate due to column instability.

1 min for the first member of the calibration series and about 8 h for the last member at a carrier gas flow rate of approximately 50 c.c./min.

This type of stationary phase has high b values for *n*-paraffins and represents difficult conditions in respect of time required for calibration.

In spite of this the minimum calibration ranges required for accuracy are well within the maximum convenient calibration ranges.

Under other conditions (polar stationary phases, normal capillary columns) calibration times are much reduced.

Summarising, under the worst conditions of separate chromatograms throughout and using a single secondary standard

$$\varepsilon_{xN} = \sqrt{2}\,\varepsilon_0 \tag{110}$$

$$\varepsilon_{x9} = 2\varepsilon_0 \tag{111}$$

That is to say the probable error for retention data in R_{x9} units should be $\sqrt{2}$ times the probable error in the conventional method.

However, the facts that the whole calibration is on a single chromatogram and that two internal *n*-paraffin standards with the unknown can be used in determining R_{x9} makes the probable error in R_{x9} considerably less.

In many cases this improvement is particularly marked for retention data in R_{x9} units and in consequence data in these units are rarely inferior in reproducibility compared with conventional methods and frequently superior (see RESULTS).

The relation between R_{x9} and the retention index

KOVATS[illegible] has defined the retention index as

$$I = 200\,\frac{\log R\,(x) - \log R\,(P_z)}{\log R\,(P_{z+2}) - \log R\,(P_z)} + 100Z \tag{112}$$

where $R(x)$ = retention of unknown
$R(P_z)$ = retention of *n*-paraffin with z carbon atoms
$R(P_{z+2})$ = retention of *n*-paraffin with $z + 2$ carbon atoms.

KOVATS does not comment on the physical significance of I. It is in fact 100 × the carbon number of a hypothetical *n*-paraffin having the same retention as the unknown. In the following discussion we replace I by I' where $I' = I/100$ since in our view I' is a more convenient parameter.

R_{x9} and I' are simply related by the expression,

$$I' = \frac{\log R_{x9}}{b} + 9 \tag{113}$$

The recommended experimental procedures for obtaining R_{x9} and I' are somewhat different, however. Thus the R_{x9} method suggested in this paper uses a number of *n*-paraffins in the calibration and therefore incorporates a check on the accuracy of the column interstitial volume apart from a generally higher standard of reproducibility which is shown in the experimental results.

The R_{x9} method is in our view more convenient for analytical purposes in that the R_{x9} value is directly proportional to elution time. In the retention index system relative elution times can only be calculated *via* a knowledge of the b factor for the column.

The retention index is reputed to have a virtually zero temperature coefficient[5], but since

$$\frac{\mathrm{d}\,I'}{\mathrm{d}\,(1/T)} = \frac{\partial I'}{\partial \ln R_{x9}}\,\frac{\mathrm{d} \ln R_{x9}}{\mathrm{d}\,(1/T)} + \frac{\partial I'}{\partial b'}\,\frac{\mathrm{d}\,b'}{\mathrm{d}\,(1/T)} \tag{14}$$

where T = absolute temperature and $b' = 2.303b$. Then from eqn. (13)

$$\frac{\mathrm{d}\,I'}{\mathrm{d}\,(1/T)} = \frac{1}{b'}\,\frac{\mathrm{d} \ln R_{x9}}{\mathrm{d}\,(1/T)} - \frac{\ln R_{x9}}{b'^2}\,\frac{\mathrm{d}\,b'}{\mathrm{d}\,(1/T)} \tag{15}$$

$$= \frac{1}{b}\,\frac{\mathrm{d} \log R_{x9}}{\mathrm{d}\,(1/T)} - \frac{\log R_{x9}}{b^2}\,\frac{\mathrm{d}\,b}{\mathrm{d}\,(1/T)} \tag{16}$$

The condition for zero temperature coefficient for I' is thus

$$b\,\frac{\mathrm{d} \log R_{x9}}{\mathrm{d}\,(1/T)} = \log R_{x9}\,\frac{\mathrm{d}\,b}{\mathrm{d}\,(1/T)} \tag{17}$$

When this condition does not hold, the temperature coefficient of I' will clearly be a function of temperature.

The thermodynamic significance of R_{x9} values

Most work of a thermodynamic nature in gas chromatography has followed thermodynamic convention for vapour–solution equilibria and taken the pure liquid solute at the temperature and average pressure of the column as the standard state[6,7].

This introduces a number of experimental complications in that to determine true activity coefficients at infinite dilution (γ^∞) the fugacities of the pure solutes must also be determined. In general this means determining the vapour pressure of the pure solutes and correcting for non-ideality of the vapours via knowledge of the molar volumes in the liquid phase and the second virial coefficients. For most compounds this data is not available. If, however, the vapour at infinite dilution is taken as the standard state it is possible to calculate from R_{x9} values (provided small samples are used) the partial molar free energy of solution increment over that for *n*-nonane at infinite dilution by the simple relation

$$\overline{\Delta G_{x9}} = RT \ln R_{x9}$$

moreover

$$\frac{\mathrm{d} \ln R_{x9}}{\mathrm{d}\,(1/T)} = \frac{\overline{\Delta H_{x9}}}{R}$$

and

$$\frac{1}{T}\,\frac{\mathrm{d} \ln R_{x9}}{\mathrm{d}\,(1/T)} - \ln R_{x9} = \frac{\overline{\Delta S_{x9}}}{R}$$

where $\overline{\Delta G}_{x9}$ = increment in partial molar free energy of solution from the gas phase at infinite dilution compared with *n*-nonane

T = absolute temperature

$\overline{\Delta H}_{x9}$ = increment in partial molar enthalpy of solution at infinite dilution from the gas phase compared with *n*-nonane

$\overline{\Delta S}_{x9}$ = associated entropy term

R = gas constant.

$\overline{\Delta H}_{x9}$ is in general sufficiently invariant with $1/T$ to make possible good temperature interpolations for R_{x9}, by plotting log R_{x9} against $1/T$.

The use of $\overline{\Delta G}_{x9}$, $\overline{\Delta H}_{x9}$ and possibly $\overline{\Delta S}_{x9}$ would appear justified by the fact that such terms are generally an additive function of chemical group contributions[8-10].

RESULTS

R_{x9} values for various solutes on squalane and dinonyl phthalate at various temperatures are shown in Tables V–IX. Alongside the R_{x9} value the standard deviation and the percentage standard deviation (obtained from at least nine determinations of R_{x9}) are quoted. The remaining columns in Tables V–IX give the conventional retention ratios taking various *n*-paraffins as internal standards. The standard deviations and percentage standard deviations of these values are also quoted.

R_{x9} values for *n*-paraffins are shown in Tables III–IV together with percentage standard deviations.

TABLE III

n-PARAFFINS ON 20% DNP/CELITE

Substance	64.7° $b = 0.377$ $P_b = 0.0009$			100° $b = 0.311$ $P_b = 0.0009$		
	R_{x9}	$P_{x9}\cdot 10^3$	σ_{x9}	R_{x9}	$P_{x9}\cdot 10^3$	σ_{x9}
n-Pentane	0.031	0.79	2.5			
n-Hexane	0.074	0.79	1.1	0.113	2.0	1.8
n-Heptane	0.176	0.79	0.45	0.237	2.6	1.1
n-Octane	0.421	1.0	0.24	0.487	2.5	0.51
n-Nonane	1.000	0.00	0.00	1.000	0.00	0.00
n-Decane	2.377	4.5	0.19	2.051	6.0	0.29
n-Undecane	5.657	15.4	0.27	4.196	16.0	0.38
n-Dodecane				8.556	43	0.50
n-Tridecane				17.52	131	0.75
	K *n*-nonane ~ $0.76\cdot 10^3$			K *n*-nonane ~ $0.22\cdot 10^3$		

It is to be noted that although the standard deviation is always low, the values vary widely. In the case of rapidly eluted materials a relatively high standard deviation is to be expected but, apart from this factor, the origin of the diversity of standard deviations is hard to explain.

The theory of the R_{x9} method suggests that owing to the fact that at elevated temperatures calibration ranges of *n*-paraffins were used which are wider than neces-

TABLE IV

n-PARAFFINS ON 20% SQUALANE/CELITE

Substance	61.7° $b = 0.311$ $P_b = 0.0007$			100° $b = 0.324$ $P_b = 0.0016$			138° $b = 0.264$ $P_b = 0.0007$		
	R_{x9}	$P_{x9} \cdot 10^3$	σ_{x9}	R_{x9}	$P_{x9} \cdot 10^3$	σ_{x9}	R_{x9}	$P_{x9} \cdot 10^3$	σ_{x9}
n-Pentane	0.027	0.73	2.7	0.050	1.3	6.6			
n-Hexane	0.067	0.75	1.1	0.105	1.3	1.3			
n-Heptane	0.165	0.84	0.51	0.224	1.8	0.79	0.305	3.3	1.1
n-Octane	0.407	1.5	0.37	0.474	2.6	0.54	0.549	3.7	0.68
n-Nonane	1.000	0.0	0.00	1.000	0.00	0.00	1.000	0.00	0.00
n-Decane	2.459	4.6	0.19	2.110	7.2	0.34	1.839	4.8	0.26
n-Undecane	—	—	—	4.446	39	0.89	3.386	8.2	0.24
n-Dodecane	—	—	—	9.410	77	0.82	6.230	20	0.33
n-Tridecane	—	—	—	—	—	—	11.48	49	0.43
n-Tetradecane	—	—	—	—	—	—	21.22	120	0.58
	K *n*-nonane ~ $1.2 \cdot 10^3$			K *n*-nonane ~ $0.31 \cdot 10^3$			K *n*-nonane ~ $0.13 \cdot 10^3$		

sary to maintain constant reproducibility of R_9, then the reproducibility should improve with increase in temperature. By taking the mean standard deviation at each temperature, for all the materials studied in this paper, this trend is evident (Table X). Also in this table are shown (1) the mean standard deviation of retention ratios using a single *n*-paraffin standard with a retention in the middle range and (2) the mean standard deviation of retention ratios using in each case as internal standard the *n*-paraffin with the closest retention to that of each substance.

In general terms the reproducibility of the R_{x9} method compares with (1) but slightly inferior to (2) as predicted on theoretical grounds.

One concludes that provided identical column characteristics pertain, R_{x9} values are generally reliable to within the limits set by a standard deviation of approximately 0.5%.

Reproducibility between laboratories would therefore appear to rest largely on the identity of column characteristics. Methods by which column characteristics can be defined are being studied in these laboratories and will be published in due course.

The importance of column characterisation cannot be overemphasised, since variations in the purity or composition of the stationary phase (either initially or due to degradation with usage), the nature of the support and the surface area/volume ratio for the stationary phase may all substantially affect column characteristics in certain respects. This is shown in Tables XI–XII.

The onset of column degradation can be very sudden in certain cases. Thus a squalane column maintained at 100° gave reproducible R_{x9} values for about 140 h. Beyond this time although the R_{x9} values for relatively non polar materials showed little change, the R_{x9} values for polar or polarisable materials showed a marked drift (Table XIII).

TABLE V

20% DNP/CELITE 64.7° $b = 0.377$ K n-NONANE $\approx 0.76 \cdot 10^3$

Substance	R_{X_9} method			n-Octane = 1.00			n-Nonane = 1.00			n-Decane = 1.00		
	R_{X_9}	$P_{X_9} \cdot 10^3$	σ_{X_9}	R_{XN}	$P_{XN} \cdot 10^3$	σ_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	σ_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	σ_{XN}
Methyl ethyl ketone	0.163	1.8	1.1	—	—	—	0.164	1.9	1.1	0.069	1.0	1.5
Butan-1-ol	0.417	4.4	1.1	—	—	—	0.418	4.1	0.97	0.176	3.0	1.7
Oct-1-ene	0.426	0.90	0.21	—	—	—	0.426	1.2	0.29	0.179	0.71	0.40
n-Butyl acetate	0.786	3.3	0.41	1.867	6.5	0.35	0.785	4.6	0.58	—	—	—
Chlorobenzene	1.267	2.6	0.20	—	—	—	1.268	1.6	0.13	0.533	2.2	0.92
Hexane-1-thiol	1.863	4.1	0.22	—	—	—	1.864	4.1	0.22	0.785	3.24	0.41
Isopropylbenzene	1.961	2.19	0.11	4.664	9.9	0.21	1.959	6.2	0.31	—	—	—
Phenyl methyl ether	2.594	5.0	0.19	6.159	24	0.39	2.590	8.9	0.34	—	—	—

TABLE VI

20% DNP/CELITE 100° $b = 0.311$ K n-NONANE $\approx 0.22 \cdot 10^3$

Substance	R_{X_9} method			n-Nonane = 1.00			n-Decane = 1.00			n-Undecane = 1.00		
	R_{X_9}	$P_{X_9} \cdot 10^3$	σ_{X_9}	R_{XN}	$P_{XN} \cdot 10^3$	σ_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	σ_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	σ_{XN}
Butan-1-ol	0.438	3.3	0.76	0.438	3.4	0.77	0.214	2.1	0.98	0.104	1.6	1.5
Ethylbenzene	1.243	4.6	0.37	1.243	2.5	0.20	0.606	2.1	0.34	0.294	2.7	0.91
Hexan-1-ol	1.952	5.0	0.26	1.949	5.9	0.30	—	—	—	0.465	2.1	0.44
2,6-Dimethyl-octa-2,6-diene	2.323	8.9	0.38	2.323	7.5	0.32	—	—	—	0.555	1.5	0.26
n-Butyl n-butyrate	2.804	8.6	0.31	2.800	5.7	0.21	—	—	—	0.669	1.0	0.15
Di-isopropyl disulphide	3.661	6.9	0.19	3.597	8.9	0.25	—	—	—	0.859	2.3	0.27
Ethyl phenyl ether	3.712	5.3	0.14	3.712	11	0.29	1.811	3.3	0.18	0.884	2.1	0.24
Octan-1-ol	8.466	19	0.22	8.396	24	0.28	—	—	—	2.003	3.6	0.18

TABLE VII

20% SQUALANE/CELITE 64.7° $b = 0.391$ K n-NONANE $\approx 1.2 \cdot 10^3$

Substance	R_{X_8} method			n-Heptane = 1.00			n-Octane = 1.00			n-Nonane = 1.00		
	R_{X_8}	$P_{X_8} \cdot 10^3$	a_{X_8}	R_{XN}	$P_{XN} \cdot 10^3$	a_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	a_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	a_{XN}
Chloroform	0.036	0.38	0.68	0.338	1.9	0.55	0.137	0.76	0.55	—	—	—
Carbon tetrachloride	0.182	0.87	0.80	0.821	4.9	0.79	0.252	2.3	0.91	—	—	—
Toluene	0.250	2.6	1.0	1.553	13	0.87	0.628	5.3	0.84	—	—	—
Oct-1-ene	0.344	2.1	0.60	2.084	14	0.69	0.847	1.2	0.15	—	—	—
Chlorobenzene	0.464	1.7	0.37	—	—	—	1.141	1.7	0.15	0.464	2.7	0.57
Methyl phenyl ether	0.790	15	1.0	—	—	—	1.057	38	1.0	0.797	13	1.0
Hexane-1-thiol	0.930	9.4	1.0	5.084	63	1.1	2.310	11	0.40	—	—	—

TABLE VIII

20% SQUALANE/CELITE 100° $b = 0.324$ K n-NONANE $\approx 0.31 \cdot 10^3$

Substance	R_{X_9} method			n-Octane = 1.00			n-Nonane = 1.00			n-Decane = 1.00		
	R_{X_9}	$P_{X_9} \cdot 10^3$	a_{X_9}	R_{XN}	$P_{XN} \cdot 10^3$	a_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	a_{XN}	R_{XN}	$P_{XN} \cdot 10^3$	a_{XN}
Oct-1-ene	0.414	2.1	0.50	—	—	—	0.415	2.4	0.58	0.196	1.8	0.89
Hexane-1-thiol	1.001	5.1	0.51	2.115	11	0.53	—	—	—	0.475	2.9	0.62
Ethyl phenyl ether	1.503	15	0.98	—	—	—	1.507	16	1.1	0.713	6.8	0.95
2,6-Dimethyl-octa-2,6-diene	1.794	5.5	0.30	—	—	—	1.800	8.4	0.47	0.850	3.4	0.4
Nitrobenzene	2.333	52	2.2	—	—	—	2.342	57	2.4	1.104	22	2.0
Octane-1-thiol	4.497	17	0.39	9.514	38	0.4	—	—	—	2.129	6.6	0.31

TABLE IX

20% SQUALANE/CELITE 138° $b = 0.204$ R n-NONANE ≈ $0.13 \cdot 10^3$

Substance	R_{18} method			n-Decane = 1.00			n-Undecane = 1.00			n-Dodecane = 1.00		
	R_{18}	$P_{18} \cdot 10^3$	σ_{18}	R_{1N}	$P_{1N} \cdot 10^3$	σ_{1N}	R_{1N}	$P_{1N} \cdot 10^3$	σ_{1N}	R_{1N}	$P_{1N} \cdot 10^3$	σ_{1N}
2,6-Dimethyl octa-2,6-diene	1.020	0.3	0.58	—	—	—	0.170	2.0	0.42	0.261	1.4	0.34
Iodobenzene	2.346	3.4	0.10	—	—	—	0.661	0.82	0.12	0.370	0.38	0.15
Di-n-butyl sulphide	2.743	8.8	0.42	1.404	0.1	0.61	—	—	—	0.444	1.5	0.43
Di-p-ethoxy benzene	3.671	107	2.6	1.084	46	2.8	—	—	—	0.304	21	4.0
p-Nitrotoluene	1.306	21	0.46	—	—	—	1.438	7.0	0.32	0.738	1.0	0.34
Di-n-butyl disulphide	0.663	23	0.26	—	—	—	2.838	4.0	0.17	1.333	1.8	0.12

TABLE X

MEAN PERCENTAGE STANDARD DEVIATIONS IN RETENTION RATIOS FOR THE MATERIALS STUDIED

Stationary phase	*Type of mean %std. deviation**	*64.7°*	*100°*	*138°*
DNP	$\overline{\sigma_{x9}}$	0.55	0.47	—
	$\overline{\sigma_{xN}}$ opt.	0.52	0.33	—
	$\overline{\sigma_{xN}}$ const.	0.50	0.50	—
Squalane	$\overline{\sigma_{x9}}$	0.78	0.73	0.59
	$\overline{\sigma_{xN}}$ opt.	0.50	0.83	0.36
	$\overline{\sigma_{xN}}$ const.	0.71	0.87	0.88

* For the meaning of these symbols see the list of symbols at the end of this paper.

It is of interest to record the R_{x9} values for the I.U.P.A.C.[11] recommended standards on dinonyl phthalate and squalane at 100°. These are shown in Tables XIV–XV. Apart from the fact that these standards are not related in any way they are clearly inadequate in that they do not cover the retention scale uniformly as shown in the last column of these Tables.

TABLE XI

EFFECT OF THE CHANGE OF STATIONARY PHASE CONCENTRATION AND ALSO THE CHANGE OF SUPPORTING SOLID PHASE ON R_{x9} VALUES

(64.7°)

Substance	*20% DNP/celite*	*5% DNP/celite*	*5% DNP/brick dust*
Methyl ethyl ketone	0.163	0.175*	0.3*
Butan-1-ol	0.417	0.537*	2.0*
Oct-1-ene	0.426	0.425	0.434
n-Butyl acetate	0.786	0.819*	1.195*
Chlorobenzene	1.267	1.254	1.249
Hexane-1-thiol	1.863	1.847	1.833
Cumene	1.961	1.932	1.910
Anisole	2.594	2.590	2.633

* Asymmetric peak.

TABLE XII

EFFECT OF THE CHANGE OF STATIONARY PHASE CONCENTRATION AND ALSO THE CHANGE OF SUPPORTING SOLID PHASE ON R_{x9} VALUES

(100°)

Substance	*20% DNP/celite*	*5% DNP/celite*	*5% DNP/brick dust*
Butan-1-ol	0.438	0.500*	21.7*
Ethylbenzene	1.243	1.216	1.214
Hexan-1-ol	1.952	2.165*	—*
D.H.M.	2.323	2.302	2.299
n-Butyl *n*-butyrate	2.804	2.837	3.09*
Di-isopropyl disulphide	3.601	3.541	3.496
Phenetole	3.712	3.677	3.716
Octan-1-ol	8.400	9.14*	—*

* Asymmetric peak.

TABLE XIII

ONSET OF COLUMN DEGRADATION WITH TIME OF USAGE AT 100° FOR 20% SQUALANE/CELITE COLUMN

Substance	R_{x9} *0–140 h*	R_{x9} *150 h*	R_{x9} *157 h*	R_{x9} *164 h*
Oct-1-ene	0.414	0.416	0.417	0.417
Phenetole	1.503	1.519	1.522	1.603
Nitrobenzene	2.333	2.357	2.417	2.673
Octane-1-thiol	4.497	4.501	4.617	4.829

TABLE XIV

R_{x9} VALUES FOR THE I.U.P.A.C. RECOMMENDED STANDARDS 20% SQUALANE/CELITE, 100°

Substance	R_{x9}	$\log_{10} R_{x9}$	$\Delta\log_{10} R_{x9}$
Methyl ethyl ketone	0.071	—1.149	
			0.322
Benzene	0.149	—0.827	
			0.157
Isooctane	0.214	—0.670	
			0.476
Cyclohexanone	0.64	—0.194	
			0.020
Cyclohexanol	0.67	—0.174	
			0.047
p-Xylene	0.746	—0.127	
			0.939
Naphthalene	6.48	0.812	

TABLE XV

R_{x9} VALUES FOR THE I.U.P.A.C. RECOMMENDED STANDARDS 20% DNP/CELITE, 100°

Substance	R_{x9}	$\log_{10} R_{x9}$	$\Delta\log_{10} R_{x9}$
Methyl ethyl ketone	0.216	—0.665	
			0.011
Isooctane	0.222	—0.654	
			0.119
Benzene	0.292	—0.535	
			0.666
p-Xylene	1.353	0.131	
			0.202
Cyclohexanone	2.155	0.333	
			0.021
Cyclohexanol	2.261	0.354	
			0.874
Naphthalene	16.92	1.228	

SYMBOLS USED

δu = correction to calculated column interstitial volume (expressed as distance on chart) required to ensure linear relation between log (retention) and carbon number in the *n*-paraffin series (applicable from *n*-butane)

R = retention distance on chart measured from the "air peak"

$\delta \log R$ = deviation of log R from extrapolated linear region of the "log plot"

R_{N9} = retention of internal standard relative to *n*-nonane

R_N = retention distance on chart of the internal standard, measured from the "air peak"

R_9 = retention distance on chart of *n*-nonane measured from the "air peak" obtained by extrapolation of "log plot"

R_{x9} = retention of material being measured relative to *n*-nonane

R_{xN} = retention of material being measured relative to internal standard

ε_x = % standard deviation in the retention distance on the chart for the material being measured

ε_N = % standard deviation in the retention distance on the chart for the standard

ε'_{xN} = % error in retention ratio due to linear drift in carrier gas flow rate using the internal standard technique

x = change in carrier gas flow rate per unit of time

F_0 = carrier gas flow rate at the time of elution of the air peak

F = carrier gas flow rate at any time

t = elution time measured from air peak

ε'_{xp} = % error in the mean retention relative to a primary standard (using two internal standards eluted either side of the material being measured) due to a linear drift in carrier gas flow rate

ε_{x9} = % standard deviation in R_{x9} when material being measured, the internal standard, and each paraffin in the calibration are all run on separate chromatograms

ε_{xN} = % standard deviation in R_{xN} when the material being measured and the standard are run on separate chromatograms

ε_{N9} = % standard deviation in R_{N9} when each paraffin in the calibration and the standard are all run on separate chromatograms

φ_9 = probable error in retention distance of *n*-nonane (obtained by extrapolation or interpolation of the "log plot") divided by the probable error in the retention distance of a *n*-paraffin which is not eluted too close to the air peak

i = the carbon number of a *n*-paraffin used in the calibration

v = the number of *n*-paraffins used in the calibration

m = the carbon number of the lowest molecular weight paraffin used in the calibration

n = the carbon number of the highest molecular weight *n*-paraffin used in the calibration

$\mathscr{N}$ = the carbon number of the n-paraffin used as the primary standard (equal to 9 if n-nonane is taken as primary standard)

b = the slope of the "log plot" $\left(i.e.\ \frac{\mathrm{d}\log R_i}{\mathrm{d}i}\right)$

R_i = retention distance on the chart measured from the air peak for a n-paraffin with i carbon atoms

$n_{\max}$ = the maximum carbon number of a n-paraffin used in the calibration which is eluted in less than 8 h on a 5 ft. × 4 mm silicone 704 column with 5% stationary phase

ε_0 = % standard deviation in retention distance on chart and assumed to be independent of retention distance for materials not eluted close to the air peak

I Kovats' retention index

$R(x)$ = Kovats' equivalent of R_x used in this paper

$R(P_z)$ = Kovats' symbol for the retention distance on the chart for a n-paraffin with Z carbon atoms

$R(P_{z+2})$ = Kovats' symbol for the retention distance on the chart for a n-paraffin with $Z + 2$ carbon atoms

I' = $I/100$

T = absolute temperature of column

b' = $2.303\,b$

γ^{∞} = activity coefficient at infinite dilution in the liquid stationary phase taking the pure solute as the standard state

$\overline{\Delta G_{x9}}$ = increment in the partial molar free energy of solution at infinite dilution over that for n-nonane taking the vapour at infinite dilution as the standard state

$\overline{\Delta H_{x9}}$ = increment in partial molar enthalpy of solution associated with $\overline{\Delta G_{x9}}$ over that for n-nonane

$\overline{\Delta S_{x9}}$ = increment in partial molar entropy of solution associated with $\overline{\Delta G_{x9}}$ over that for n-nonane

P_{x9} = observed standard deviation in R_{x9}

P_b = observed standard deviation in b

σ_{x9} = observed % standard deviation in R_{x9} using internal standard technique

P_{xN} = observed standard deviation in R_{xN} using internal standard technique

σ_{xN} = observed % standard deviation in R_{xN} using internal standard technique

$\overline{\sigma_{x9}}$ = mean percentage standard deviation in R_{x9} for all the values quoted in this paper

$\overline{\sigma_{xN}}$ opt. = mean percentage standard deviation in R_{xN} using a n-paraffin as internal standard with the closest retention to each material being measured for all the values quoted in this paper

$\overline{\sigma_{xN}}$ const. = mean percentage standard deviation in R_{xN} using one n-paraffin as internal standard with a retention in the middle range of retentions measured at each temperature and stationary phase.

SUMMARY

The reproducibility of retention data in R_{x9} units is discussed theoretically. It is shown that high reproducibility should be readily obtainable by using suitable calibration procedures and experimental evidence in support of this is presented for a wide range of compounds.

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THE FLAME IONIZATION DETECTOR
A THEORETICAL APPROACH

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(Received December 27th, 1960)

The flame ionization detector of McWILLIAM AND DEWAR[1, 2] still lacks a comprehensive explanation of the various electrical and chemical phenomena observed. An attempt is made in the present paper to provide at least partial explanations of some of these phenomena by specific mechanisms.

The detector is, in general, sensitive to organic gases and vapours, and insensitive to the common inorganic gases and vapours. It can now be regarded as fairly certain that the response to organics is specifically associated with the carbon atoms. Although the process of ion formation is chemically very inefficient (perhaps 1 in 10^5) the ion collection efficiency can apparently be 100% efficient and the ion currents obtainable are more than adequate for most practical purposes of detection.

It is proposed to discuss the mechanisms of the detector under three headings:

1. Site of ion formation and charge separation.
2. Electric field effects in ion collection.
3. Mechanism of ion formation.

SITE OF ION FORMATION AND CHARGE SEPARATION

The flame burns either pure hydrogen or a mixture of an inert gas and hydrogen, usually in air, and is regarded as comprising three zones of conceptual importance, *viz.*, a gap between the jet and the base of the flame, where no combustion takes place; an ionization and charge separation zone probably corresponding to the inner cone; and the main volume of hot gases above the inner cone. The metal jet serves as one electrode, and a collecting electrode above the flame as the other.

Several considerations lead to the idea that the site of ion formation is a thin layer at the base of the flame. The insensitivity of the detector to carbon monoxide and dioxide suggests that the upper part of the flame will contribute nothing to ion formation from organic molecules, because the latter can hardly be supposed to survive as such for any distance at a temperature in the vicinity of 2000°, and surrounded by an atmosphere rich in oxygen and water vapour.

At the inner cone, however, the temperature is suddenly raised from ambient to well over 1000°, and in this very restricted region it is supposed that the rare event of ion formation takes place.

If an electric field is now applied between the jet and the collector, charge separation takes place at the site of ion formation. Ions of one polarity then cross the cold gap and reach the jet, those of the opposite polarity remain in the flame initially, and eventually may be collected (possibly after chemical re-arrangement or exchange in the flame).

These concepts, if true, lead to several interesting consequences. Let us suppose, for convenience, that the jet is positive and that the field applied is sufficient to cause complete separation at the site of formation of all ions formed. Positive ions now inhabit the upper part of the flame without corresponding negative ions (electrons) with which to recombine, and consequently their residence time before collection does not affect the total ion current. They do, however, constitute a space charge which will modify the electric field in proportion to their concentration. In high concentrations they will also, by mutual repulsion, tend to fan outwards and some may miss the upper electrode altogether if it does not subtend a large enough angle at the jet tip. Finally, if charge separation is to take place at the base of the flame, then this is the place where the electric field should, if possible, be concentrated for the best effect.

The hypothesis that formation of ions takes place in a thin zone at the inner cone of the flame is supported by the work of KINBARA AND NAKAMURA, as quoted by ONGKIEHONG[3], on hydrocarbon flames.

The results of changing the shape of the collector electrode in such a way that the electric field is concentrated around the jet tip, as discussed below, are regarded as supporting the idea that the base of the flame is the site of ion formation and charge separation.

ELECTRIC FIELD EFFECTS

A considerable variety of collector electrode shapes have been used by various workers—rods, wires, rings and gauzes, for example. At adequate voltages these give sensitive and sometimes linear responses to small amounts of organics. MCWILLIAM[4] has shown, however, that the geometry of the upper electrode is very much concerned with extending linear response into the high concentration region. In his work he used a gauze square and found that if this was placed sufficiently close (5 mm) to the jet the linear range could be extended to concentrations of ethylene at least of several per cent, whereas if the electrode was raised much above 5 mm the response fell off progressively from linear as the concentration increased, indicating a complete loss of some of the ions available.

These effects can be explained by the mutual repulsion of the ions as discussed above. At the author's suggestion, MCWILLIAM tried the effect of a cylindrical hat-shaped gauze electrode. Although placed so that the crown of the hat was much more than 5 mm above the flame, this gave results as good as those obtained with the close flat electrode, the sides of the hat preventing the escape of mutually repelling ions. The lower edge of the cylinder was approximately level with the tip of the jet. As a rough guide, it seems that the upper electrode periphery should subtend an angle of at least 90° at the tip of the jet in order to get linear response over the whole range of concentrations up to at least 5 % of a light gas. Still higher concentrations can be tolerated with an upper electrode of optimum design, as discussed below. The voltage required for linear response with a suitable electrode increases with sample concentration, but no practicable increase of voltage will compensate for an electrode design which is inherently inadequate for high concentrations.

The shape and position of the upper electrode also affects the relations between current and voltage at voltages below that required for maximum current, and these relations are not the same when the polarity is reversed, as shown by DESTY *et al.*[5] at low sample concentrations and by MCWILLIAM[4] at high ones. DESTY has suggested that the polarity differences are related to the relative mobilities of the positive ions and electrons and the consequent differences in space charge effects. This is undoubtedly true, but DESTY found a curious kink in the curves when the jet is positive which is not explained, although he has speculated that it may be caused by two different species of positive ion.

In the author's view the kink and other features of the voltage–response curves can be explained entirely by the effects of space charge on the distribution of the electric field. As mentioned above, the efficiency of charge separation is regarded as a function of the field intensity immediately above the jet. Now with a jet consisting of a thin tube there is, in the absence of space charge, a concentration of field just above the tip, as shown schematically in Fig. 1a. The effect of a space charge formed in the main body of the flame will be a tendency to lift the equipotential lines away from the tip and to distribute them more evenly in the space between the two electrodes. The first incidence of space charge as the voltage is increased from zero thus affects the field at the crucial point rather sensitively. When the space charge has built up to the point where the field is more or less uniform between the electrodes, further increase of voltage causes an approximately linear increase in the field and the response rises again to saturation. The kink is not observed when the jet is negative because the much higher mobility of the electrons reduces the space charge in the body of the flame, although as DESTY's curves show, there is a small effect from the electron space charge which is shown up by changing the geometry of the upper electrode. If the upper electrode is optimally designed the saturation voltage with positive jet is always less than with negative jet, as will be shown, and this is believed to be explained by the difficulty of moving the massive positive ions counter-current to the gas stream flowing at about 5 m/sec at the jet exit.

Experimentally, the concept of field distortion by the geometry of the electrodes has been supported by the author's experiments with collector electrodes designed to exaggerate the distortion up to a practical limit. These electrodes consisted of gauze cylinders of various diameters, closed at the top, and placed so that the jet, in the extreme case of the narrowest cylinder, was well up inside the cylinder. It was reasoned that the equipotential lines should be distributed in the manner shown schematically in Fig. 1b. Using a steady sample concentration from *n*-hexane at dry ice temperature, a carrier gas of 50/50 N_2/H_2 mixture and a 23 gauge hypodermic needle tube for the jet, the minimum positive jet voltage required for saturation current decreased from 70 for a flat disc 1 cm above the jet to 10 for the narrowest cylinder, and the kink, which was very evident with the flat disc electrode, only showed as a slight decrease in slope.

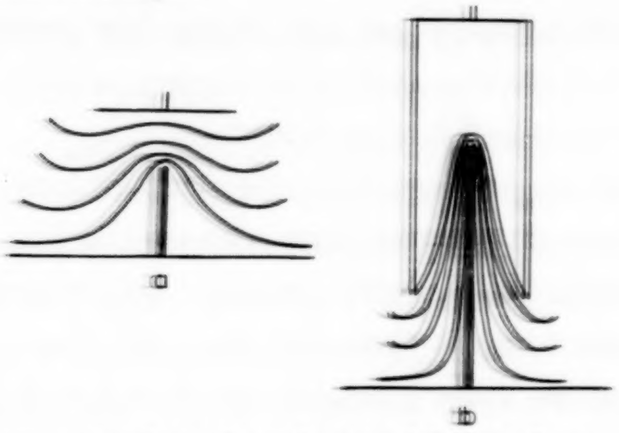

Fig. 1. Schematic representation of equipotential lines for two electrode shapes.

If the ions formed in the reaction zone are regarded as randomly distributed in velocity and direction then those with high velocities in the right direction should be easily extracted and those with high velocities in the wrong direction should be difficult to extract. The overall effect on response, as voltage is increased from zero, and in the absence of space charge effects, should correspond to the integral of the Gaussian function. In Fig. 2, a plot of this integral from tabulated values, and adjusted to fit at zero and at saturation, is shown superimposed on the response curve using the long narrow cylinder electrode. The fit is exact up to half saturation, and the deviation above this point is taken to be a measure of the residual space charge effect. At lower concentrations, the fit is better still.

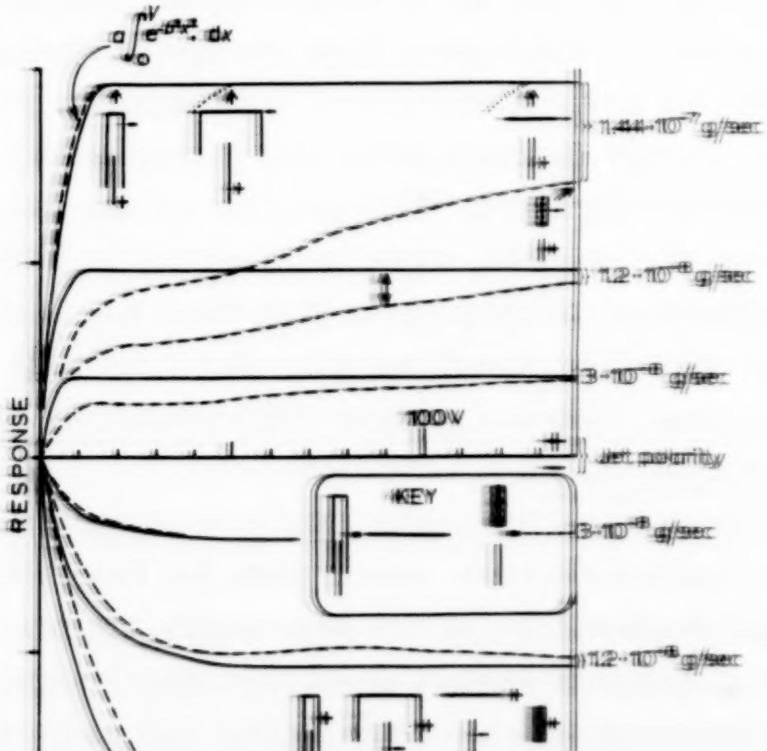

Fig. 2. Response *vs.* voltage for narrow cylindrical gauze electrode (solid lines) and vertical rod (broken lines) for three concentrations. Saturation voltages required for other electrodes also indicated, and relation to Gaussian function shown for one curve.

An interesting question arises with hollow cylindrical electrodes, namely the fate of ions which have passed through the equipotential line corresponding to the upper electrode potential itself, and have thus entered the Faraday cage region. It might be thought that a large dead volume in the Faraday cage would cause a lag in response time, but actually an ion which has entered the cage is already effectively collected as far as the electrical circuit is concerned, and its physical contact with the metal can take place at leisure.

It follows from this reasoning that the narrow hollow cylindrical electrode should indeed have the fastest response time because the upper electrode (represented in effect by its equipotential line) is very close to the jet and hence very close to the reaction zone. The response time in terms of the resolution of a series of spike pulses of sample will depend on the time required to clear the field of the ions formed at each pulse. The clearance function should be Gaussian and theory would indicate that resolution should improve as voltage is increased, even above that required for saturation. In practice, the resolution is usually governed not by the ion collection rate but by the time constant of the electrical circuit, which is quite appreciable with multi-megohm resistors and unavoidable stray capacitance. The residence time in the reaction zone of organic molecules entering with the carrier gas stream is quite short. Thus, if the inner cross section of the jet is 0.1 mm^2 and the gas rate is 30 c.c./min, the issuing velocity is 5 m/sec. If the reaction zone is say 1 mm deep, (it may be much less) the residence time is thus about 0.2 msec, during which a sample molecule must either form an ion pair or be destroyed without yield.

In Fig. 2 a comparison is made of the response *vs.* voltage curves, at three moderately low concentrations in the range $3 \cdot 10^{-8}$ to 10^{-7} g/sec, for electrodes of two extreme types, *viz.* a flat-ended vertical rod and a narrow gauze cylinder, each for positive and negative jet polarities. It will be seen from the traces that although the rod is as good as the cylinder for low concentrations (with negative jet), its performance falls away remarkably at high concentrations and it requires much higher voltages to achieve the true saturation current. With positive jet the rod produces kinks on the ascending portion of the response curve, as observed by DESTY. With negative jet, kinks are also produced at voltages higher than those which at first sight produce the saturation current and the current actually decreases with increasing voltage over a certain voltage range. This is a genuine and reproducible effect only observed with the rod and is perhaps explained as follows:

At low to moderate voltages the vertical surfaces of the rod, for some distance above the base plane, collect electrons which miss the base plane. As the voltage rises still further, the collection area moves downwards towards the base plane and a "mushroom" of space charge is formed about the latter. Some of the outer fringe of electrons are thus deflected outwards still further and are too far removed from the vertical surfaces for collection: they thus escape altogether.

At higher sample concentrations, the narrow gauze cylinder with positive jet is far better than all other shapes tried, both with respect to the lower voltages required for saturation current and the extension of linear response to very high concentrations. For capillary column work, 20 V would suffice in most cases, for samples up to a few micrograms. In the range above 1 % sample concentration at the detector, response appeared sensibly linear up to 17 % ethylene, the highest concentration tried, at a voltage of 320. Few, if any, katharometers give linear response in these high ranges. No other electrode system tried showed any possibility of reaching saturation current in the highest range, at least without extremely high voltages. The precision of the linear relationship has not been measured over the complete range of concentrations, taken as a whole, from the lowest to the highest. To check this would require accurate chemical and electrical calibrations at the extreme ends of the concentration scale on the same instrument. Although perhaps few people would expect to extrapolate over a range of 10,000 to 1, the utility of being able to do so is considerable, since the major components can be used to calibrate the minor ones.

Fig. 3 shows the voltages required for saturation current for the rod and narrow cylinder electrodes over the concentration range from 10^{-8} to 10^{-4} g/sec for positive and negative jet potentials. Wide cylinders, and discs, with positive jet potential, give curves intermediate between the extremes of the rod and narrow cylinder but

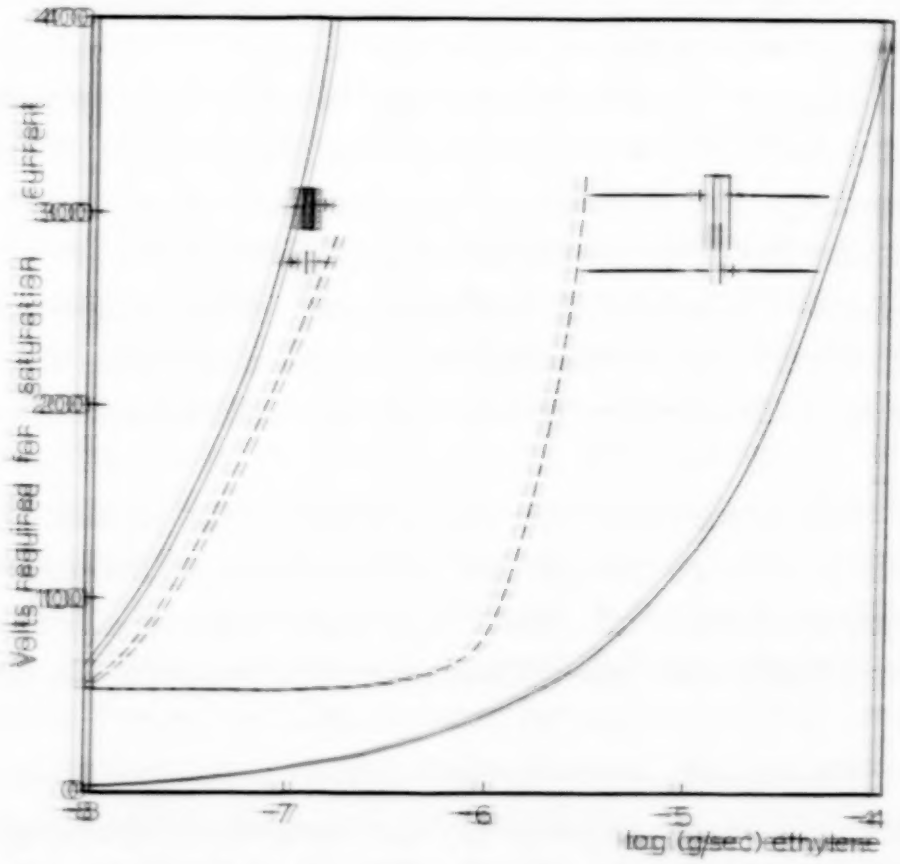

Fig. 3. Sample rate *vs.* voltage required for saturation current.

with negative polarity they give initially almost identical flat portions (at 40 V) up to about 10^{-7} g/sec, which then turn sharply upwards in the same manner as the narrow cylinder curve, but at a concentration about a factor of 2 lower. The flat initial portion of these curves, showing independence of concentration, represents the counter-current effect mentioned above. The sharp turn upwards must represent the onset of space charge effects. The sharpness of the upturn, as compared with the more gradual rise when the jet is positive, may possibly be accounted for by the fact that although electron diffusion is not much hindered by the presence of gas, electron motion upwards is also not much assisted by the upward flow of the gas. The opposite holds for the positive ions, which are assisted in their upward passage by the gas stream. Total gas velocity should thus affect the shape of the curves, including effects due to the velocity of the ventilation air.

MECHANISM OF ION FORMATION

It is evident from MÜLLER's work[7] that the electric field itself cannot produce ions in the flame detector from any ordinary molecule with an ionization potential of the order of 10 eV, since this would require a field of the order of 100 MV/cm, while the available field in the flame is usually only a few hundred volts per cm.

The problem then is to find a thermal or thermochemical mechanism which is specific for carbon atoms which are initially in particular environments (organic molecules). The hydrogen possibly contributes nothing but a high temperature. Hydrocarbons alone burning in air give large ion yields. On the other hand, carbon monoxide has been shown in this laboratory to be useless as the combustion gas and to give a very low background current, while in a hydrogen flame it is said by ONGKIEHONG[3] to give a weak signal.

The very wide range of linearity observed with the hydrogen flame ionization detector seems in the author's view to exclude on the grounds of reaction kinetics any mechanism requiring the collision or co-operation of even two carbon atoms per ionization event, let alone the formation of a large assembly. It might be possible to concede a two-carbon reaction if methane and other single-carbon molecules behaved differently from those containing two or more carbons, but this is not observed, and methane is as sensitively detected at low concentrations as are the higher hydrocarbons.

We are driven to suppose then that all or most of the carbon atoms in organic molecules have each a certain though low probability of reacting to form an ion pair, and that the reaction must take place, if it takes place at all, during a residence time between about a tenth and one millisecond at a temperature between ambient and about 2000°.

There are few clues regarding the nature of the ion formation process. We know that simple inorganic gases and vapours do not yield ions, that organic substances in general do give responses, including carbon tetrachloride and dichlorodifluoromethane, and that organic molecules containing carbon linked to oxygen show some relative reduction in ion yield. Thus ONGKIEHONG[3] suggests a rule for discounting such carbons in calculating ion yields.

There are two exceptions, recently discovered in this laboratory, to the general rules regarding inorganic and organic responses. MCWILLIAM[4] has found, contrary to the usually accepted finding, that carbon disulphide does give a response at very low concentrations, although of a very peculiar kind limited by a unique self-suppressing effect at higher concentrations. Also, the author has found that formic acid gives no measurable response even from the saturated vapour at room temperature (about 2 %). These two liquids, together with water, thus offer three convenient solvents for introducing trace samples without interference to the record).

The discovery of the lack of response to formic acid followed from a theoretical speculation that formic acid might be the species responsible for the primary ion-forming reaction, by chemical dissociation into formate-ion and a proton aided by the high exothermic reaction of the proton with a water molecule to form H_3O^+. If this hypothesis were true, the response to formic acid vapour should have shown a considerable enhancement. The complete lack of response, taken in conjunction with observations regarding the reduced response of other partly oxidised molecules, suggests that the energy of oxidation of the carbon atoms is in some way connected with the ionization phenomenon. In formic acid, the carbon is as highly oxidised as it can be, short of forming carbon dioxide.

If we consider the changes in bond energies involved in forming carbon dioxide from carbon atoms, disregarding completely the bond energies of any links broken in the process of oxidation, we have—from values listed by COTTRELL[6], the following rough picture:

Hydrocarbon C: Full bond energy of $CO_2 = 2 \times 192 = 384$ kcal/mol
Alcohol C: $384 - 85.5 = 298.5$ kcal/mol
Ketone C: $384 - 179 = 205$ kcal/mol
Carboxyl C: $384 - 85.5 - 179 = 119.5$ kcal/mol

We know from the formic acid experiment that carboxyl carbons contribute nothing, in one case at least. We also have the observations of ONGKIEHONG and others that ketone carbons must be either partly or wholly discounted. Carbon monoxide should be in a similar category on the above theory, and in fact gives only a weak response, according to ONGKIEHONG. Methanol gives a sensitive response, probably less, however, than methane on a molar basis. Carbon tetrachloride on this hypothesis should give the same molar response as methane, whereas in fact it gives probably about half this: the rule can thus not be applied in any strict quantitative sense, but it may be an approximation to the truth and indicate the order of energy required in the ion formation. The reasons for neglecting the energy of other bonds broken in the oxidation process are firstly that in the present state of knowledge the available quantitative data are not adequate for more than rough qualitative speculation and secondly that DESTY's results for benzene and cyclohexane, which gave almost identical molar responses, suggest that bonds (other than to oxygen) can vary considerably without a very marked effect. The quite sensitive response of carbon tetrachloride indicates lack of necessity for any special kind of pre-existing bond, *e.g.* C—H or C—C. It may be that carbon atoms destined to be involved in ion formation are already stripped of one or more of their attachments by thermal cracking. The essential step in ion formation may be an electronic transition of low probability in an excited CO_2 molecule, the energy of excitation being provided by the oxidation process.

If complete oxidation of carbon atoms in a short time in a restricted space is a pre-requisite to ion formation, the physical accessibility of the carbon atoms to collision by high velocity oxygen molecules, leading to a one-step formation of CO_2, may be of importance. Thus methyl groups would be more accessible than CH_2 groups, and tertiary and quaternary carbons would be perhaps in effect inaccessible. If the probability of reaction is favoured by greater accessibility to oxygen attack, then one might expect differences in sensitivity due to this factor to be decreased when oxygen is in free supply and to be accentuated under conditions of oxygen starvation. Desty's[5] results for eleven hydrocarbons have been examined to see whether a correlation can be found between physical accessibility and response, with results that appear to be interesting though not conclusive. The series is too short for sufficient independent checks of the quantitative theory, which had to be evolved step by step by working through the list, making "rules" to suit the observed responses. The fit is thus partly or wholly Procrustean in all except one case. Nevertheless, the "rules" seem logically defensible, qualitatively speaking, albeit the arguments may sometimes seem rather fine-spun. This series is the longest available to the author in which the measurements are all expressed in absolute terms and independent of integration errors and of reference to relative values.

Starting the series with the straight chain C_6, C_7 and C_8 hydrocarbons, it was assumed that the methyl groups would contribute more than the CH_2's. The calculation can thus be performed in terms of the "apparent CH_3 value" of each carbon, giving this a value unity for CH_3 itself. The observed responses for this series were 0.510, 0.579 and 0.640 coulombs/mole respectively. By trial and error, the apparent CH_3 value of the CH_2's was found to be approximately 0.5 for a good fit. Thus we have

n-Hexane: $2 + (4 \times 0.5) = 4\ CH_3$: 0.1274 coulombs/$CH_3$
n-Heptane: $2 + (5 \times 0.5) = 4.5\ CH_3$: 0.1286 coulombs/$CH_3$
n-Octane: $2 + (6 \times 0.5) = 5\ CH_3$: 0.1280 coulombs/$CH_3$

Average 0.1280 coulombs/CH_3

The next in the series are cyclohexane, methylcyclohexane, and ethylcyclohexane, which had responses 0.492, 0.537, 0.603 coulombs/mole. If we apply the rule as above (for $6 \times 0.5 = 3\ CH_3$) to cyclohexane, the coulombs/CH_3 obtained is much too large, *viz.* $0.492 \div 3 = 0.1640$. Now benzene, with response almost identical at 0.495, would show the same effect, and we are required empirically to add an average of 0.85 CH_3 to the 3 CH_3 for cyclohexane and benzene giving 3.85 CH_3 for each, and yielding coulombs/CH_3 0.1278 for cyclohexane, 0.1286 for benzene.

The extra CH_3 value is accounted for in the theory by a "bending outward effect", all the ring carbons being less capable of concealment by their neighbours, as compared with the CH_2's in the straight chain compounds, which in certain foldings are more concealed by their neighbours than are the ring carbons. The value 0.85 is equivalent to 0.142 CH_3 per ring carbon: each ring carbon thus is valued at 0.642 CH_3 groups. Passing now to methylcyclohexane, and only introducing the new concept that the tertiary carbon is valueless, we have the following:

5 carbons at 0.5 each = 2.5
5 carbons at 0.142 each = 0.71 (bending outward effect)
1 carbon at 1 = 1.00 (CH_3)
1 carbon at 0 = 0.0 (tertiary C)
Total 4.21 CH_3: coulombs/CH_3, 0.1273
Deviation from 0.1280, —0.55 %

Similarly for ethylcyclohexane, we have

5 carbons at 0.5 = 2.5 (ring)
5 carbons at 0.142 = 0.71 (bending outward effect)
1 carbon at 0.5 = 0.5 (side chain CH_2)
1 carbon at 1.0 = 1.0 (CH_3)
1 carbon at 0 = 0.0 (tertiary C)
Total 4.71 CH_3: coulombs/CH_3, 0.1277
Deviation from 0.1280, —0.23 %

(This is the only compound in the whole series for which new concepts were not necessary.)

The aromatics were puzzling, with benzene practically indistinguishable from cyclohexane, but toluene and ethylbenzene not showing nearly the increases expected for the side chains. The respective responses were 0.495, 0.508, 0.535 coulombs/mole. On the accessibility hypothesis, it seemed that the aromatic ring was shielding the side chain from collision with oxygen molecules much more than the ring in the cyclohexane series. Assuming that this is so, can one explain the considerable shielding effect on the second carbon in the side chain in ethylbenzene? A model shows that for most of the time this carbon is in fact out of the plane of the ring and is shadowed by it over a considerable solid angle, but why the aromatic ring should do this and not the aliphatic ring is another matter. This might be explained by the greater thermal stability of the aromatic ring, which would enable it to remain intact for a longer time, implying also that the cyclohexane ring is sometimes at least already partly disintegrated at the crucial moment. The effect of 30 % oxygen added to the hydrogen by DESTY, which obliterated the relative reduction in response of toluene compared with *n*-heptane, may thus be explained by the increase in flame temperature, or by the greater chances of a suitable collision in the presence of ample oxygen, so that shielding effects become statistically less rate-determining.

Toluene can be pulled into line by multiplying the value for the side chain carbon by a shielding factor of 0.75. Thus we have

Toluene: 5 × 0.642 = 3.21 (ring + bending outward)
1 × 0.75 = 0.75 (shielded CH_3)
1 × 0 = 0.0 (tertiary C)
3.96 CH_3: coulombs/CH_3, 0.1280

In the case of ethylbenzene, the shielding factor for the CH_2 is required by the observed response to be greater still, the suggestion being that the ring shadow is greater for an out-of-plane attachment. The factor required is 0.635.

Ethylbenzene: 5 × 0.842 = 4.21

1 × 0.635 = 0.635 (out-of-plane shielded CH_2)

[illegible] × 0.635 = [illegible] (in-plane shielded CH_3)

1 × 1.00 = 1.00 (terminal C)

[illegible] CH_2 (observed CH_2 [illegible])

Turning finally to the two highly branched paraffins, 2,2,3-trimethylbutane and 2,2,4-trimethylpentane, with response factors [illegible] and [illegible] [illegible] respectively, we find enhanced responses as expected, corresponding to the larger number of methyl groups, each having five. The actual response of 2,2,3-trimethylbutane is, however, about 7% lower than expected for five methyl groups (ignoring the quaternary and tertiary carbons). A model shows that the methyl groups are so crowded together that their hydrogen atoms could be imagined to have an appreciable mutual shielding effect on the adjacent methyl carbons. An average deduction of 7% in the value of each methyl group is required to account for the deficiency in response.

The response of 2,2,4-trimethylpentane is, of course, [illegible] higher than expected for five methyl groups plus one CH_2. In this molecule the methyl groups are much less crowded, and also the CH_2 is always "between methyls". Allowing for the latter, we have

5 × 1.0 = 5 (normal CH_3)

1 × 0.842 = 0.842 (shielding normal CH_2)

2 × 0 = 0 (tertiary and quaternary C)

5.842 CH_2 (observed CH_2 [illegible])

Whatever the true explanation of response variations may be, it is evident that under the conditions of DESTY's detector the "rules" are likely to be inconveniently complicated for practical use in the precise prediction of response factors. On the other hand, ONGKIEHONG's results seem to fit simpler rules in some cases. DESTY himself found that adding oxygen to the hydrogen decreased the discrepancy between toluene and *n*-heptane. It is not perhaps unreasonable to suppose, on the accessibility hypothesis, that the "rules" may be simpler when oxygen is in very free supply. DESTY's burner consisted of a rather massive platinum tip which would restrict access of air from below the orifice, as compared with a hypodermic needle. Also, he normally used pure hydrogen which has both a very high flame velocity and outward diffusion rate, so that again the flame would cover the jet at the tip over a wide area and render difficult the access of oxygen to the inner cone. It would be interesting to have a comparable and preferably longer series done with a very fine "fish-tail" jet designed for maximum access of air to all parts of the flame at the base, or with a needle jet with Bunsen effect. The improvement in response obtained by the addition of nitrogen when using needle jets may be explained likewise by greater accessibility of oxygen at the base of the flame because the lower flame velocity would lift the base of the flame off the jet, as indeed it visibly appears to do when sufficient organic substance is present to make the flame visible.

It is to be hoped that more data, expressed in absolute terms, will become available, especially on the effect of burner design and gas composition on the simplicity or otherwise of sensitivity relationships, and on the effect of oxygen bonds in various environments on absolute responses. The transition in sensitivity in passing from the lower to the higher members of homologous series would also be most useful in the construction of a rational theory.

ACKNOWLEDGEMENT

The author is grateful to Mr. V. E. MAIER for his efficient planning and carrying out of the experimental work described.

SUMMARY

An analysis is presented of the reasons for various changes in the response of the flame ionization detector with respect to electrode geometry and molecular structure of sample species. The electrode geometry affects the distribution of the electric field at the base of the flame, where ionization and charge separation are believed to occur, and also affects the incidence of space charge effects on the electric field. The hypothesis is put forward that one-step oxidation of carbon atoms to CO_2 is essential in ion formation, and that molecular geometry affects the probability of this occurring.

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NOMOGRAMS FOR GRADIENT ELUTION CHROMATOGRAPHY

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(Received December 12th, 1960)

Gradient elution chromatography, as reported by ALM *et al.*[1] and DONALDSON *et al.*[2] has provided new facilities for the separation of mixtures and is widely used. The concentration, ionic strength, or other characteristics of the eluting agent, are continually changing in the course of the experiment. The change is usually nonlinear and the determination of the particular characteristic at a given moment of the experiment, for instance at the peak, is difficult. The theoretical approach to this problem[1,3,4] has led to equations that make possible the computation of the relationship between the characteristics studied and the volume of the eluting agent, under given experimental conditions and in given solutions. This elaborate computation has to be repeated after every change in the working conditions.

In this paper it is shown that some relations can be replaced directly by nomograms. However, in some cases the quantities that are necessary for drafting the nomogram have to be obtained from auxiliary nomograms. The application of nomograms is always time-saving and facilitates the choice of optimal experimental conditions.

This paper deals with the gradients of concentration and ionic strength, the latter being proportional to the concentration at constant pH value.

THEORETICAL

I. A single closed mixing chamber

This simple case is widely used although it gives a less advantageous convex gradient. The equation[1,4,5] giving the dependence of the concentration on the volume of the eluting agent may be written as follows:

$$\frac{c_1 - c}{c_1 - c_2} = e^{-v/V_2} \tag{1}$$

and transformed to a determinant

$$\begin{vmatrix} 0 & xc_1 & 1 \\ \delta & xc_2 & 1 \\ \frac{1}{D}\left[x\delta\left(e^{-v/V_2} - 1\right)\right] & \frac{1}{D}\left[-x\beta c\right] & 1 \end{vmatrix} = 0$$

where: $D = (\alpha - \beta)e^{-v/V_2} - \alpha$, c_1 is the concentration of the agent in the reservoir, c_2 the initial concentration of the agent in the mixing chamber, V_2 the volume of the mixing chamber, c the concentration and v the volume of the agent flowing into the column. α, β, δ are scale moduli, conveniently chosen as 10, 10, and 20 cm, respectively. By comparing this determinant with a general one

$$\begin{vmatrix} \xi_1 & \eta_1 & 1 \\ \xi_2 & \eta_2 & 1 \\ \xi & \eta & 1 \end{vmatrix}$$

the design equations can be given. For example, the design equation for c_1 is derived from the first row of the determinant: $\xi_1 = 0$, $\eta_1 = 10c_1$. The scale for c_2 can be computed from the second row. The third row contains the quantity c as well as the ratio v/V_2 and is represented by a network, situated between the scales c_1 and c_2.

The nomogram is represented in Fig. 1. It is used as follows: given values for the initial concentration in the reservoir, c_1, and in the mixing chamber, c_2, are connected by a straight line. The intersection of this straight line with a vertical line cutting the corresponding mark on the scale for v/V_2 gives the actual concentration. When repeating a series of experiments with identical starting conditions, the connecting line between c_1 and c_2 is drawn in pencil.

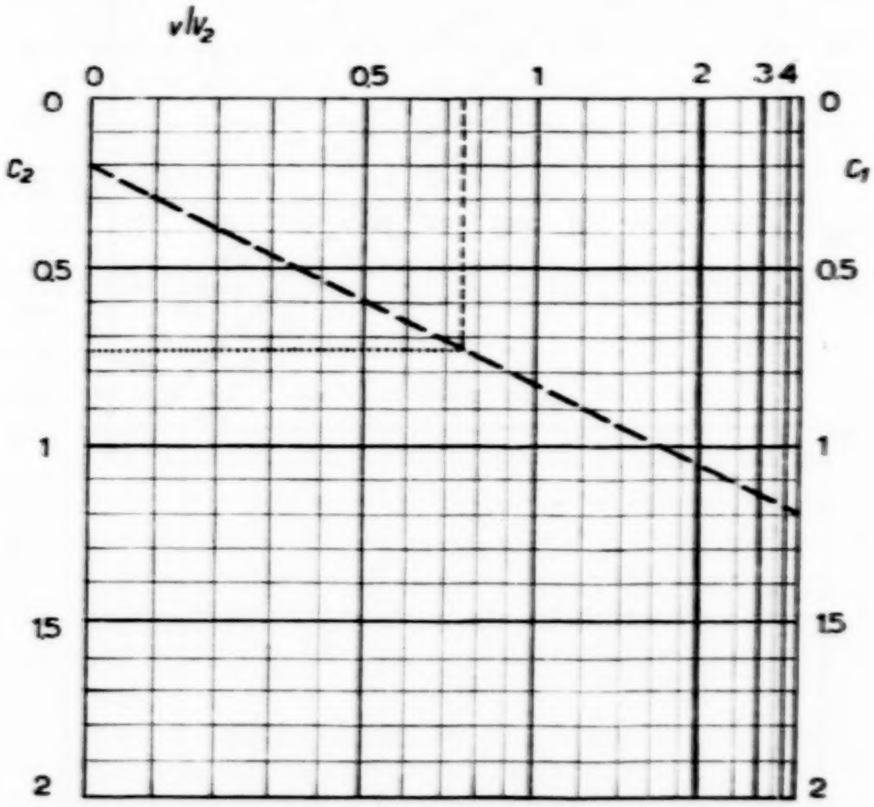

Fig. 1. Nomogram for a closed mixing chamber.

As an example, the computation of one case with starting conditions $c_1 = 1.2\ M$, $c_2 = 0.2\ M$ and $v/V_2 = 0.75$, is given in Fig. 1. The result is read on the vertical scale as $c = 0.73\ M$.

II. Reservoir and mixing chamber as communicating vessels

(*a*) *The difference in the densities is small.* For this relatively simple and universal method of forming a gradient, equations have been derived[5], that are similar in form to eqn. (1) for a closed mixing chamber. With cylindrical vessels we have

$$\frac{c_1 - c}{c_1 - c_2} = \left(1 - \frac{v}{V_t}\right)^{\varkappa} \tag{2}$$

where V_t is the sum of the starting volumes of reservoir and mixing chamber and $\varkappa = q_1/q_2$ is the ratio of the cross-sections of both vessels. Compared with eqn. (1), eqn. (2) differs in the function of volume. Hence the computation of the nomogram is the same, except that the horizontal scale for v/V_t is different. Let this scale be denoted as x and calibrated uniformly from zero to 1 in the direction of the scale from c_1 to c_2.

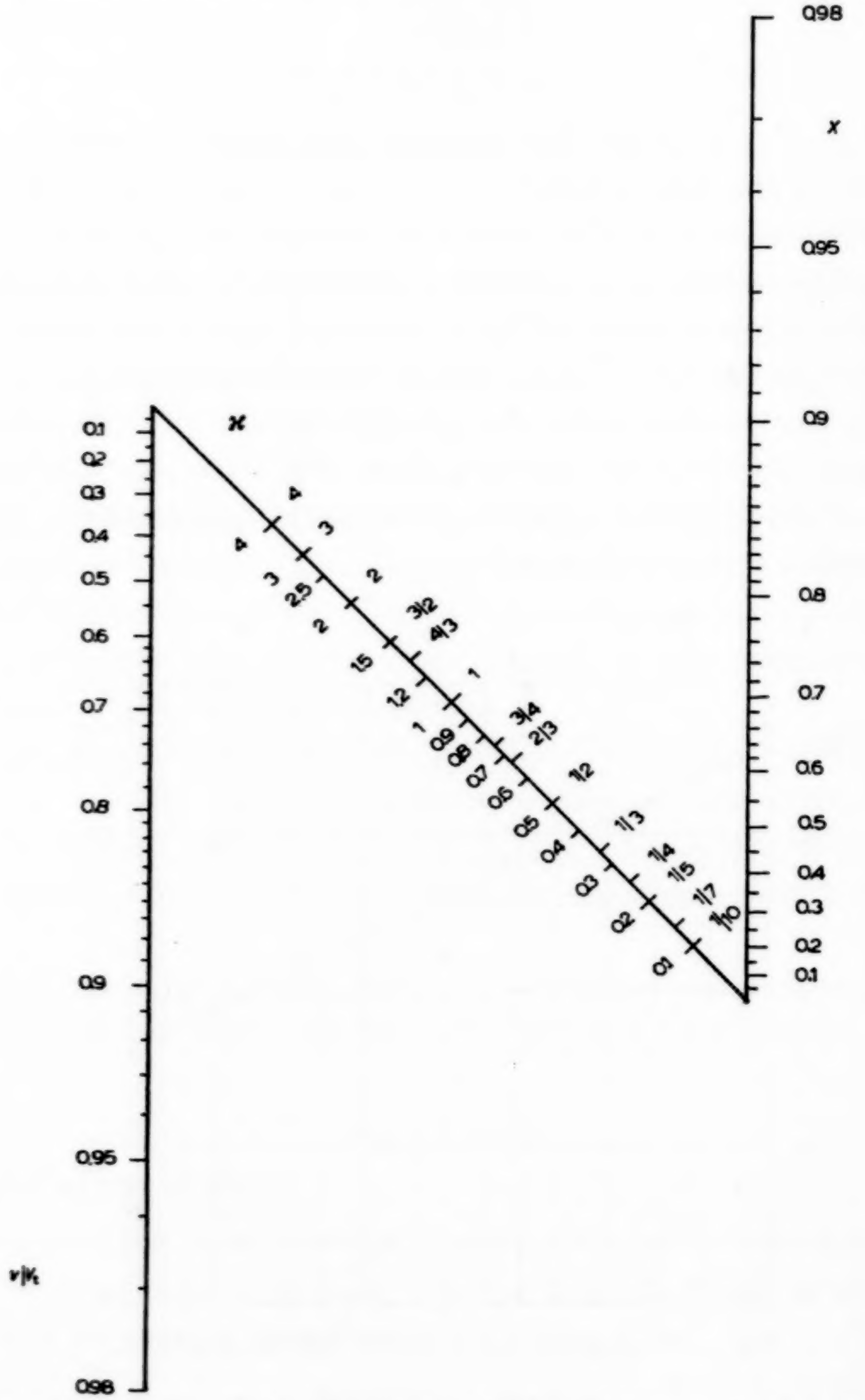

Fig. 2. Nomogram for computing the function $x = 1 - (1 - v/V_t)^{\varkappa}$.

The nomogram in Fig. 2 gives the relation of x and v/V_t and $\varkappa$. The value of $\varkappa$ for a given experimental arrangement, read on the inclined scale, is connected with the particular value for v/V_t by a straight line. When extended, this line, subtends the corresponding value for v/V_t on the scale x. Fig. 3 is an auxiliary diagram for marking the scale v/V_t with whole tenths. On a strip of paper placed horizontally at the height

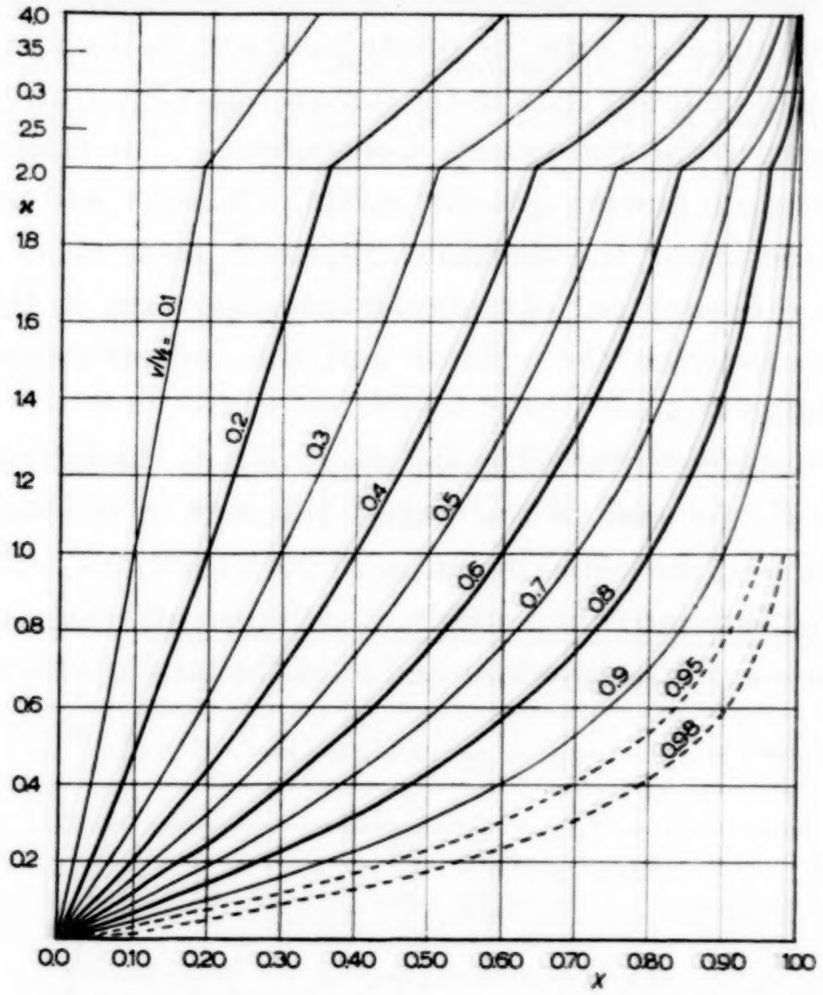

Fig. 3. Graph of the function $x = 1 - (1 - v/V_t)^\varkappa$ for definite values of v/V_t.

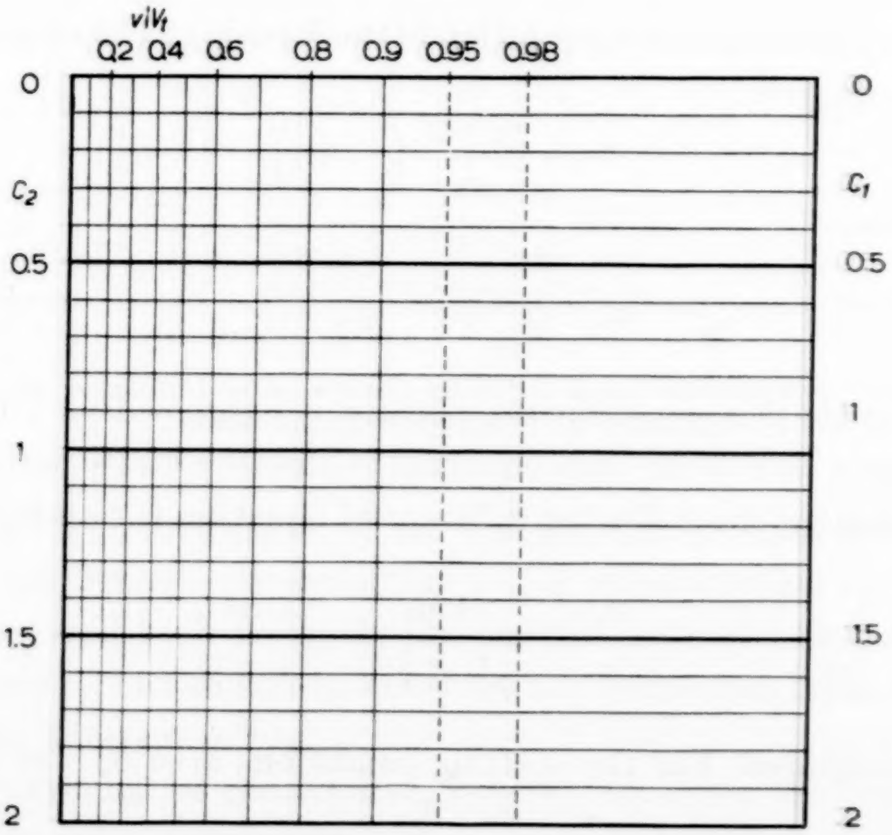

Fig. 4. Nomogram for communicating vessels. Ratio of cross-sections 1 :4. The solutions in both vessels are of the same density.

of the corresponding value for $\varkappa$ are drawn the intersections with the curved scales for particular values of v/V_t. From this auxiliary strip the scale is transferred to the nomogram. Fig. 4 is an example of this technique for $\varkappa = 1/4$.

The nomogram is used in the same way as the nomogram in Fig. 1.

(*b*) *The densities differ widely*. If the solution in the reservoir is fairly concentrated, then after it has communicated with the dilute solutions in the mixing chamber, the level in the reservoir will be lower than in the mixing chamber, owing to the difference in density of the liquids in the two vessels. Consequently, the relative volume of the more concentrated solution flowing into the mixing chamber will be smaller. During the course of the experiment the densities approach each other and the relative volume of the solution flowing out of the reservoir converges to the value $\varkappa$. Let us compute the relation between the volume and the concentration of the solution flowing out for the latter case.

Let c_1 be the initial concentration in the reservoir, c_2 the initial concentration in the mixing chamber, V_t the sum of the initial volumes in the reservoir and in the mixing chamber, s_1 and s_2 the initial densities of both solutions, v the total volume of agent that has flowed out into the column, c its concentration, and s its density. For the actual volumes v_1 in the reservoir and v_2 in the mixing chamber we have

$$v_1 + v_2 = V_t - v$$

and

$$v_1 : v_2 = \frac{q_1}{s_1} : \frac{q_2}{s} = \varkappa : \frac{s_1}{s}$$

Whence

$$v_1 = \frac{\varkappa}{\varkappa + s_1/s}\left(V_t - v\right), \quad v_2 = \frac{s_1/s}{\varkappa + s_1/s}\left(V_t - v\right), \quad \mathrm{d}v_1 = -\frac{\varkappa \cdot \mathrm{d}v}{\varkappa + s_1/s}$$

The change of concentration of the solution in the mixing chamber may be represented by a differential equation

$$\mathrm{d}c = \frac{c_1 - c}{v_2}\left(-\mathrm{d}v_1\right)$$

Substitution gives

$$\frac{\mathrm{d}c}{(c_1 - c)\cdot s/s_1} = \frac{\varkappa \cdot \mathrm{d}v}{V_t - v}$$

In order to integrate this equation the density is expressed as a linear function of the concentration $s = 1 + kc$. This equation is almost exactly satisfied.

After substitution the following differential equation is obtained

$$\frac{\mathrm{d}c}{c_1 - c} + \frac{k \cdot \mathrm{d}c}{1 + kc} = \frac{\varkappa \cdot \mathrm{d}v}{V_t - v}$$

which can be integrated. For the starting conditions $v = 0$, $c = c_2$ we have

$$\frac{c_1 - c}{c_1 - c_2} \cdot \frac{1 + kc_2}{1 + kc} = \left(1 - \frac{v}{V_t}\right)^{\varkappa}$$

or after introducing the densities

$$\frac{c_1 - c}{c_1 - c_2} \cdot \frac{s_2}{s} = \left(1 - \frac{v}{V_t}\right)^{\varkappa} \tag{3}$$

This equation differs from eqn. (2) merely by the factor s_2/s on the left-hand side. In order to transform this equation into a nomogram let the expression on the right-hand side of the equation be designed as x. Then we transform to the determinant

$$\begin{vmatrix} 0 & \alpha c_1 & 1 \\ \beta s_2 & \alpha c_2 & 1 \\ \beta s x & \alpha c & 1 \end{vmatrix} = 0$$

The scale is marked by means of the design equations, the resulting nomogram being of the same type as that in Fig. 5. It is a deformed nomogram like that in Fig. 4. The scale c_1 is identical to that in Fig. 4, while the scale for c_2 is in general curved, but almost linear. At every point of the scale c_2 the distance from the scale c_1 is proportional to the density corresponding to the concentration c_2. The binary scale for c and v/V_t is given by a network, the inclined lines of which are geometrically similar to the scale c_2. The similarity constant x is read either from the nomogram in Fig. 2 or from the diagram in Fig. 3.

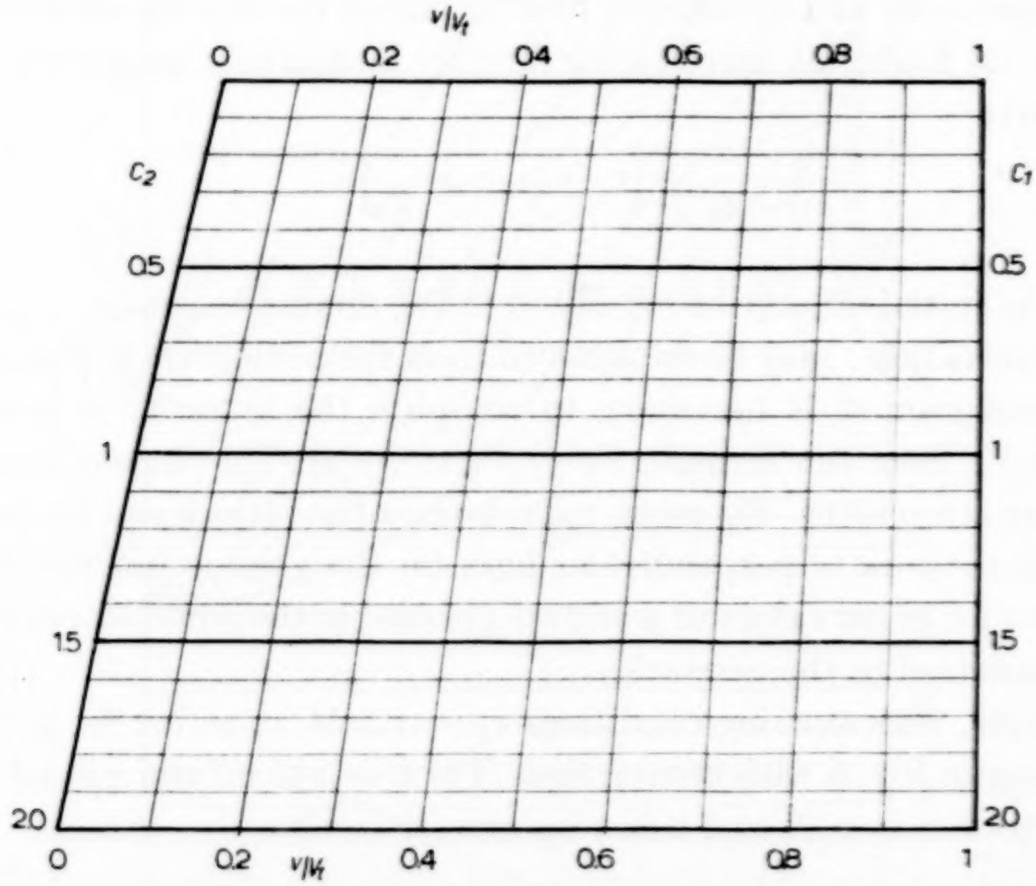

Fig. 5. Nomogram for communicating vessels with the same cross-section, containing phosphate buffer solutions of different densities.

The nomogram must be constructed, not only for every new value of the ratio $\varkappa$ (*i.e.* when changing the experimental arrangement), but also when a new buffer is used, since in general the dependence of the density on the concentration changes.

So far as these dependences are linear or at least mutually proportional a correction factor for the concentration scale may be introduced and the nomogram may be used for minor series.

III. The reservoir and mixing chamber communicate by means of a micropump

If the reservoir and the mixing chamber communicate by means of micropumps, then neither the density of the solutions nor the form of the vessels are of consequence and it is only important to maintain a uniform velocity of flow. Let r_1 be the velocity of the liquid flowing out of the reservoir into the mixing chamber, r_2 the velocity of the liquid flowing out of the mixing chamber into the column, and ρ the ratio of these velocities. The relationship between ρ and $\varkappa$ can be expressed as follows

$$\rho = \frac{r_1}{r_2}, \quad \varkappa = \frac{\rho}{1 - \rho}$$

For the concentration of the solution flowing out of the mixing chamber at a given moment, eqn. (2) holds. By introducing directly measurable quantities the equation is transformed to

$$\frac{c_1 - c}{c_1 - c_2} = \left[1 - (1 - \rho)\frac{v}{V_2}\right]^{\rho/(1 - \rho)} \tag{4}$$

where V_2 is the initial volume of the liquid in the mixing chamber.

The concentration c may be determined from the nomogram in Fig. 4. In order to draft this nomogram it is necessary to compute the quantity $\varkappa$ from the above equation and V_t from the formula $V_t = V_2/(1 - \rho)$. The nomogram in Fig. 6 is, however, more convenient. By using micropumps the ratio ρ can be easily changed and hence the network of perpendicular lines for the volume function is not drawn. The quantity x for given values of ρ and v/V_2 is read in the annexed auxiliary diagram and then transferred to the network.

An example, with starting conditions $c_1 = 1.2\ M$, $c_2 = 0.2\ M$, $\rho = 0.25$, $V_2 =$ 200 ml, is given in Fig. 6 with broken lines. For $v = 150$ ml and 220 ml respectively, the concentration $c = 0.46$ and 0.66 respectively results.

IV. Comments

1. When determining the volume v it is necessary to subtract the volume of the column and tubing between the mixing chamber and column from the volume that has flowed out of the column since the beginning of the gradient elution.

2. The computation of the relative volume is facilitated by choosing a simple value for the volume in the denominator of the fraction. In practice this can be very easily done for the cases treated in Sections I and III, *i.e.* in eqns. (1) and (4).

3. The volume V_t in eqns. (2) and (3) is correct only in the case when the bottom of the reservoir is exactly at the same level as the bottom of the mixing chamber. Since this condition is by no means necessary for the production of a given gradient,

it is not necessary to observe it when assembling the apparatus. The quantity V_t is computed from the formula

$$V_t = V_2\left(1 + \varkappa \frac{s_2}{s_1}\right)$$

where V_2 is the initial volume of the solution in the mixing chamber.

4. Nomograms can also be conveniently used in the preparation of phosphate buffer solutions of a given ionic strength[6,7].

EXPERIMENTAL

Experiments for the verification of eqn. (3) were performed in an apparatus similar to that reported by Šebesta and Schneider[8]. We do not recommend effecting the communication of the reservoir with the mixing chamber by means of a siphon, for this deforms the course of the gradient, especially at the beginning of the experiment.

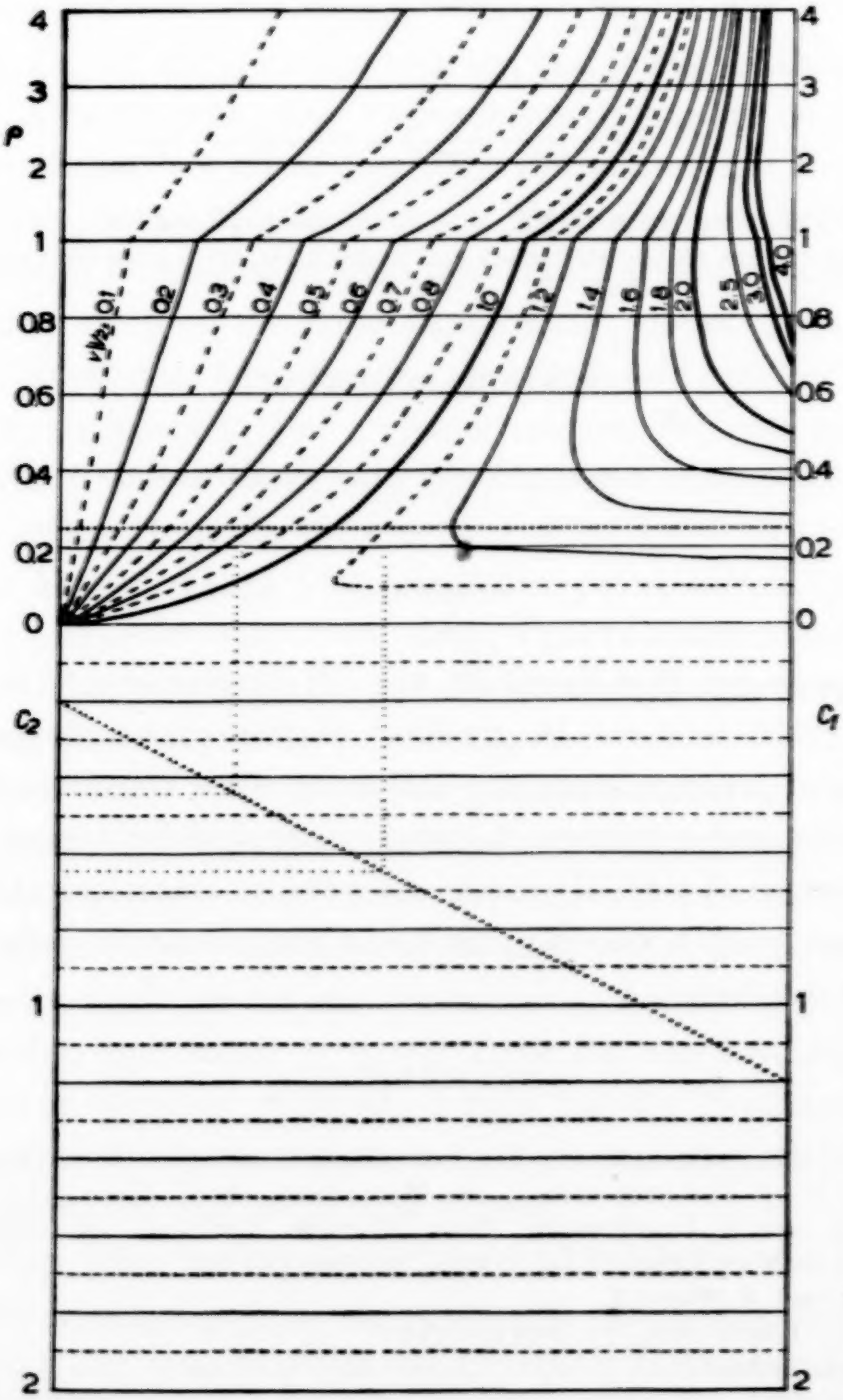

Fig. 6. Nomogram for vessels connected by means of a micropump.

The reservoir was filled with a 2 M phosphate buffer solution, coloured with acid fuchsine, and the mixing chamber was filled with a 0.1 M buffer solution prepared by diluting the first mentioned buffer. The concentration of the effluent buffer solution was determined photometrically using a calibration curve. There was good agreement between the experimental and the calculated values of the concentration (Fig. 7).

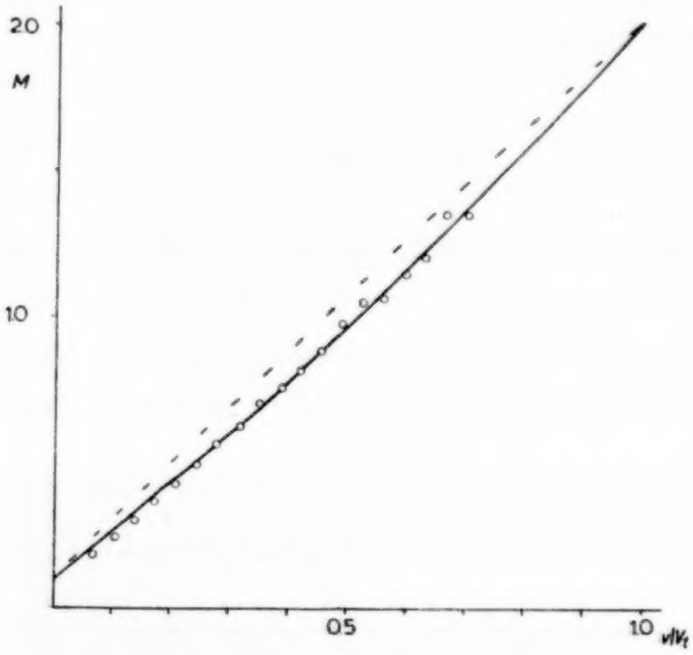

Fig. 7. Verification of the nomogram in Fig. 5. —— theoretical values; – – – – theoretical values when the differences in the densities are neglected; o = experimental readings.

ACKNOWLEDGEMENT

The author wishes to express his thanks to Miss A. ZDRÁHALOVÁ for careful drawing of the nomograms.

SUMMARY

Nomograms are suggested that facilitate the determination of the concentration of the eluates at a given moment in gradient elution chromatography. Among the various experimental arrangements the following were considered: a single mixing chamber, a reservoir and a mixing chamber connected by means of a micropump, and a reservoir connected with a mixing chamber as communicating vessels. In the latter case the relationship between volume and concentration with respect to density differences was established.

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ÜBER EINE VEREINFACHUNG DER QUANTITATIVEN BESTIMMUNG VON AMINOSÄUREN AN AUSTAUSCHERSÄULEN MIT DER MÖGLICHKEIT GLEICHZEITIGER QUALITATIVER UNTERSUCHUNGEN*

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(Eingegangen den 10. Januar 1961)

Die verschiedenen Methoden der Säulenchromatographie und der Papierchromatographie sind die z. Zt. am meisten verwendeten und leistungsfähigsten Verfahren zur Bestimmung von Aminosäuren. Die Papierchromatographie ist für die qualitative Analyse von Aminosäuren vor allem durch die Einfachheit der Handhabung und den geringen apparativen Aufwand allen anderen Methoden gleich guter Trennwirkung überlegen. Sie wurde in vielen Fällen auch zur quantitativen Bestimmung von Aminosäuren herangezogen, doch ist die Leistungsfähigkeit dieser Methode hier noch sehr begrenzt und die Fehlerbreite noch zu gross, um eindeutig gesicherte und reproduzierbare Werte liefern zu können. Bei Untersuchungen, die eine exakte quantitative Bestimmung erfordern, ist es daher angebracht, der Säulenchromatographie als der z. Zt. leistungsfähigsten Methode auf diesem Gebiet den Vorzug zu geben und hier besonders der von MOORE, STEIN UND SPACKMAN entwickelten Chromatographie an Ionenaustauschern entweder mit Hilfe eines Fraktionssammlers[2] oder aber unter Verwendung der vollautomatischen Apparatur der gleichen Autoren[3] bzw. der vereinfachten Ausführung dieser Methode von HANNIG[4].

Der Nachteil all dieser säulenchromatographischen Verfahren liegt darin, dass keine Möglichkeit besteht, neben der quantitativen Bestimmung der einzelnen Gipfel einer Elutionskurve auch eine qualitative Prüfung auf ihre Identität und Einheitlichkeit durchzuführen bzw. unbekannte Komponenten zu identifizieren, da die gesamte Menge jeder Fraktion für die quantitative Auswertung restlos verbraucht wird. Diese Methoden sind daher in erster Linie für die Bestimmung von Aminosäuren in Proteinhydrolysaten geeignet, jedoch für die Bestimmung unbekannter Naturprodukte, z.B. freier Aminosäuren in biologischem Material, bei denen Identitätsprüfungen unerlässlich sind, nicht ausreichend.

Wir entwickelten daher auf der Grundlage des Verfahrens von MOORE, SPACKMAN UND STEIN[2] eine chromatographische Apparatur, die die Möglichkeit bietet, in einem Trennvorgang ausser der quantitativen Bestimmung auch eine spezielle qualitative

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* Siehe auch Lit.[1].

Untersuchung eines jeden Elutionsgipfels durchzuführen. Darüber hinaus wird durch eine weitgehende Vereinfachung der quantitativen Auswertung der Fraktionen eine so erhebliche Einsparung an Zeit und Reagenzien erzielt, dass mit dem sonst für eine Analyse notwendigen Aufwand mehrere Analysen nebeneinander durchgeführt werden können.

Das Prinzip der Methode besteht darin, dass jede 1-ml-Fraktion des Eluates einer Austauschersäule mit Hilfe eines elektronischen Programmreglers alternierend in eine Test- und eine Hauptfraktion zerlegt wird; die Testfraktionen werden halbquantitativ getestet und die Hauptfraktionen auf Grund dieser Testung im Bereich jedes einzelnen Gipfels zu jeweils einer Gesamtfraktion zusammengefasst. Die quantitative Bestimmung erfolgt in einem aliquoten Teil jeder Gipfelfraktion, so dass anschliessend ausreichende Mengen für weitere Untersuchungen zur Verfügung stehen. Die Anzahl der quantitativen Einzelbestimmungen verringert sich auf diese Weise von *ca.* 300–600 auf die Zahl der vorhandenen Gipfel, also auf etwa 20 Bestimmungen.

METHODISCHES

Säule

Die chromatographische Trennung wird an Ionenaustauschern durchgeführt und zwar an Amberlite IR 120 (CG 120) mit einer nach dem Flotationsverfahren von Hamilton[5] ausfraktionierten einheitlichen Korngrösse von *ca.* 50 µ. Die Säulenlänge beträgt für die Bestimmung der sauren und neutralen Aminosäuren 160 cm, für die Bestimmung der basischen Aminosäuren 25 cm; der Durchmesser der Säule ist in beiden Fällen 0.9 cm. Die Rohrfüllung erfolgt nach den Angaben von Moore, Spackman und Stein[2], ebenso die Elution mit den von den gleichen Autoren verwendeten Pufferlösungen. Die Untersuchungssubstanz wird in einer Menge von 0.2–0.3 µ Mol je Aminosäure oder 1–3 mg hydrolysierten Proteins in einer Lösung von 0.1–1 ml im pH-Bereich 2.0–2.5 auf die Säule gegeben. Die Trennung erfolgt bei einer Temperatur von 50°, die durch einen Umlaufthermostaten konstant gehalten wird. Die sauren und neutralen Aminosäuren werden mit 0.2 *N* Na-Citratpuffer eluiert und zwar zunächst mit Pufferlösung vom pH 3.25 bis zum Erscheinen von Prolin, dann mit Pufferlösung vom pH 4.25 bis zum Erscheinen der letzten Aminosäure. Die Elution der basischen Aminosäuren wird mit Pufferlösung vom pH 5.28 mit einer 0.35 *N* Na-Konzentration vorgenommen.

Um zu verhindern, dass sich innerhalb der Säule Luftblasen bilden, die die Säule unbrauchbar machen würden, werden die Pufferlösungen zur Entfernung gelöster Luft gekocht, heiss mit Paraffinöl überschichtet und bei 4° aufbewahrt.

Zur Einhaltung einer konstanten Elutionsgeschwindigkeit von *ca.* 15 ml/h wird auf die Pufferlösung, die sich in einem Vorratsgefäss befindet, ein Druck von etwa 30 cm Hg gegeben. Die Zuführung des Druckes erfolgt entweder direkt aus einer Stickstofflasche mit Reduzierventil oder bei mehreren parallel laufenden Säulen zweckmässigerweise aus je einem kleinen, etwa 3 l fassenden und mit Stickstoff bis zum gewünschten Druck gefüllten Metallbehälter, aus dem dann der Druck über einen ausreichend langen Zeitraum gleichmässig abgegeben werden kann.

Um eine fortlaufende Untersuchungsfolge zu gewährleisten, werden bei uns die benutzten Säulen in einer besonderen Regenerationsanlage regeneriert. Hier können bis zu 12 der 160-cm-Säulen nebeneinander aufgehängt und durch Hindurchschicken von 100 ml 0.2 *N* NaOH und 100 ml Pufferlösung vom pH 3.25 zur weiteren Verwendung bereit gemacht werden. Die zur Trennung der basischen Aminosäuren verwendeten 25-cm-Säulen werden mit 20 ml 0.35 *N* NaOH und anschliessend 100 ml Pufferlösung vom pH 5.28 regeneriert.

Fraktionierung

Die Fraktionierung des Säuleneluates erfolgt durch Tropfenzählung mit Hilfe eines Streifkontaktes (Fig. 1). Die Tropfen fallen hierbei durch ein System frei nach unten hängender Platindrähte und stellen dadurch kurzzeitig einen Kontakt her. Die Impulse werden über ein elektronisches Relais einem Zählwerk zugeführt, das dann den Transportmechanismus des Fraktionssammlers auslöst.

Für die Steuerung des Wechsels zwischen Test- und Hauptfraktionen wurden zwei verschiedene Geräte zur elektronischen Programmregelung entwickelt. Das erste Gerät – Zählerform A – ähnelt im Prinzip der Anordnung einer Telefonwählanlage und ermöglicht Variationen der Tropfenzahl zwischen 0 und 9 Tropfen für die Testfraktion und zwischen 0 und 119 Tropfen für die Hauptfraktion. Das zweite Gerät –

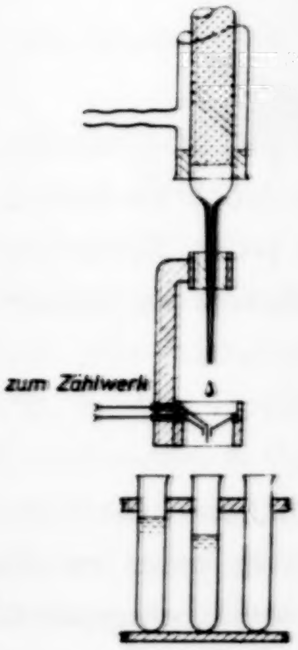

Fig. 1. Tropfenzählung durch Streifkontakt.

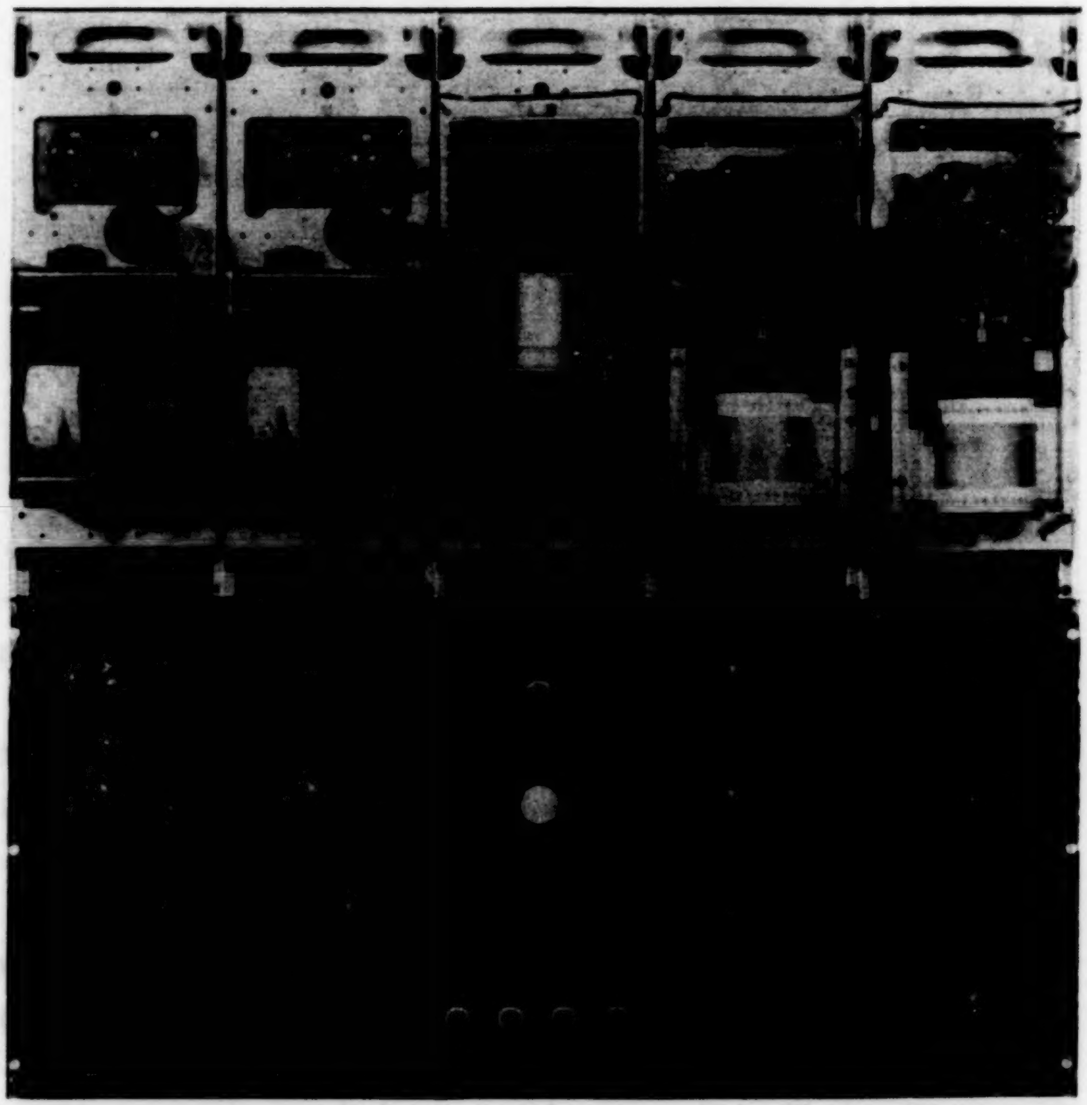

Fig. 2. Steuerungsgeräte für 4 Fraktionssammler. Links: 2 Geräte der Zählerform B. Rechts: 2 Geräte der Zählerform A. Mitte: Das Netzgerät.

Zählerform B – ist demgegenüber erheblich vereinfacht; es besteht aus 8 Scheiben, die eine jeweils verschiedene Anzahl von Schaltnocken tragen und so eingestellt werden können, dass die Hauptfraktion nach Wahl 7, 12, 17, 21, 27, 37, 57 und 117 Tropfen beträgt – das ist zwischen 1/4 und 3 ml – während die Testfraktion konstant 3 Tropfen erhält. Die Stromversorgung der Zähler erfolgt über ein zentrales Netzgerät.

Die Fig. 2 zeigt die Steuerungsgeräte der Zählerformen A und B mit dem in der Mitte liegenden Netzgerät. Bei der Zählerform A (Zähler 3 und 4) wird die gewünschte Anzahl der Tropfenfolge für die Test- und Hauptfraktion auf den darunter liegenden Schaltskalen eingestellt; bei der Zählerform B (Zähler 1 und 2) erfolgt die Einstellung der Tropfenzahl – in diesem Falle nur für die Hauptfraktion, da die Testfraktion konstant 3 Tropfen erhält – durch Verstellen des Kontaktarmes der Nockenscheiben am Zähler selbst. Die Einsteckleisten sind so angeordnet, dass jeder Zählertyp wahlweise für jeden Sammler eingesetzt werden kann. Das Schaltschema der Anlage ist in Fig. 3 dargestellt.

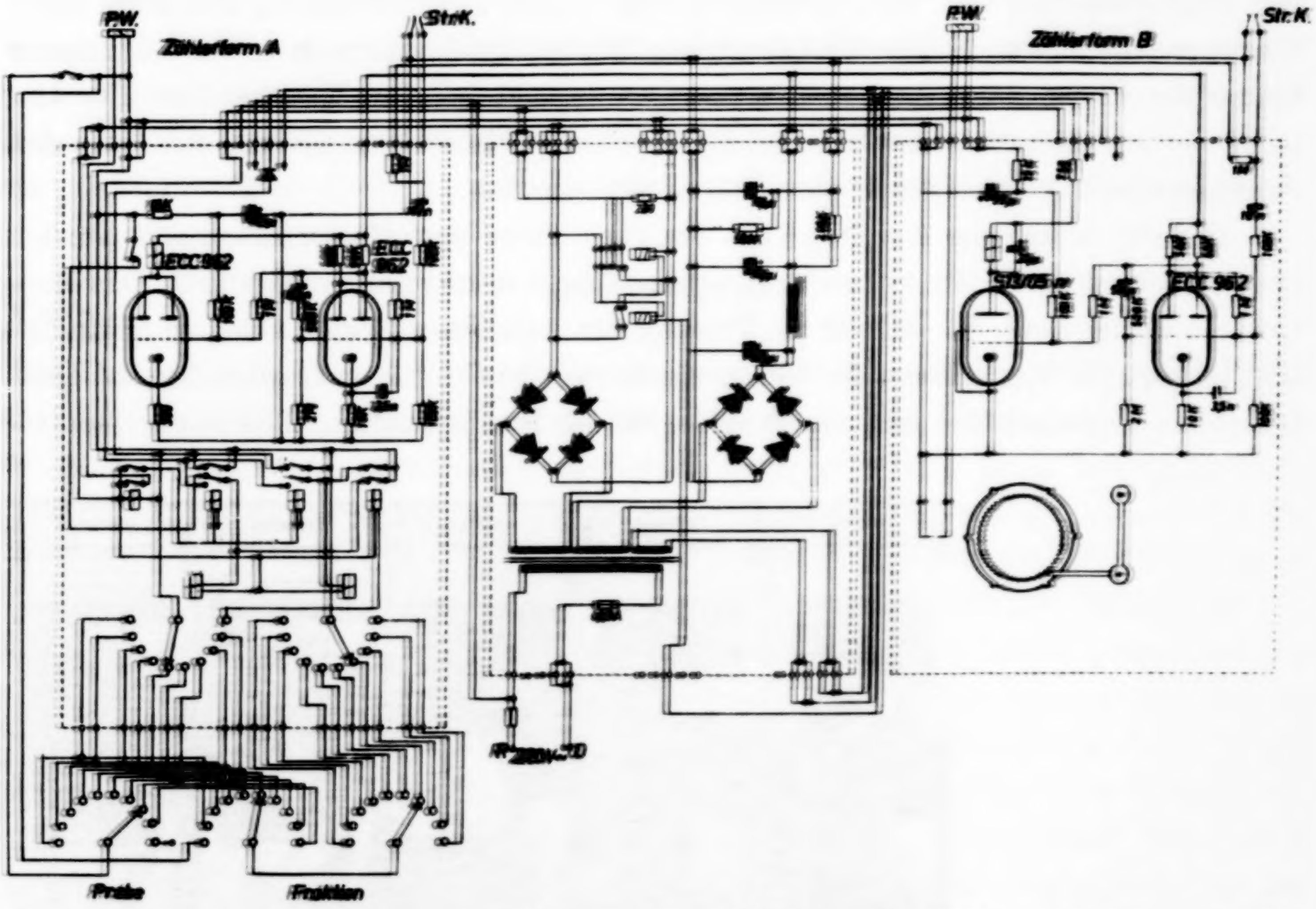

Fig. 3. Schaltschema der Steuerungsanlage. Str. K. = Streifkontakt. P.W. = Fraktionssammler.

Fraktionssammler

Zum Auffangen der Fraktionen ist im allgemeinen jeder der üblichen Fraktionssammler geeignet. Er wird mit kleinen Reagenzgläsern beschickt; in jedes zweite Glas wird ein kleines Spitzgläschen zur Aufnahme der Testfraktionen eingesetzt. Fig. 4 zeigt 4 Fraktionssammler mit 4 Austauschersäulen, die zu einem Aggregat zusammengefasst sind, mit den dazugehörigen Steuerungsgeräten.

Vortestung

Die Spitzröhrchen mit den Testfraktionen – im allgemeinen 3 Tropfen Eluat – werden entsprechend der Fraktionsfolge dem Sammler entnommen und in ein Gestell mit einem Aufnahmevermögen von 200 Gläsern eingesetzt. Nach Zugabe von 2 Tropfen Ninhydrinreagenz werden die Röhrchen in dem Gestell 20 Minuten in einem siedenden Wasserbad erhitzt. Durch visuelles Schätzen der entstandenen Farbintensität lässt sich eine halbquantitative Elutionskurve aufstellen, ähnlich der oberen Kurve in Fig. 6. Die tropfenweise Zugabe des Ninhydrinreagenz erfolgt aus einer braunen Tropfflasche, in der das Reagenz aus Ninhydrin und Hydrindantin[6] direkt hergestellt wird und zwar zur Vermeidung schädigender Lufteinwirkung mit Hilfe eines Magnetrührers unter einer Paraffinölschicht, unter der es dann ständig verbleibt. Eine U-förmige Kapillare und ein Glasrohr, das zum Schutz vor Verunreinigungen in einer kleinen Erweiterung mit Watte gefüllt ist, führen durch einen doppelt durchbohrten Stopfen, mit dem die Flasche verschlossen ist, bis auf den Boden der Flasche bzw. in

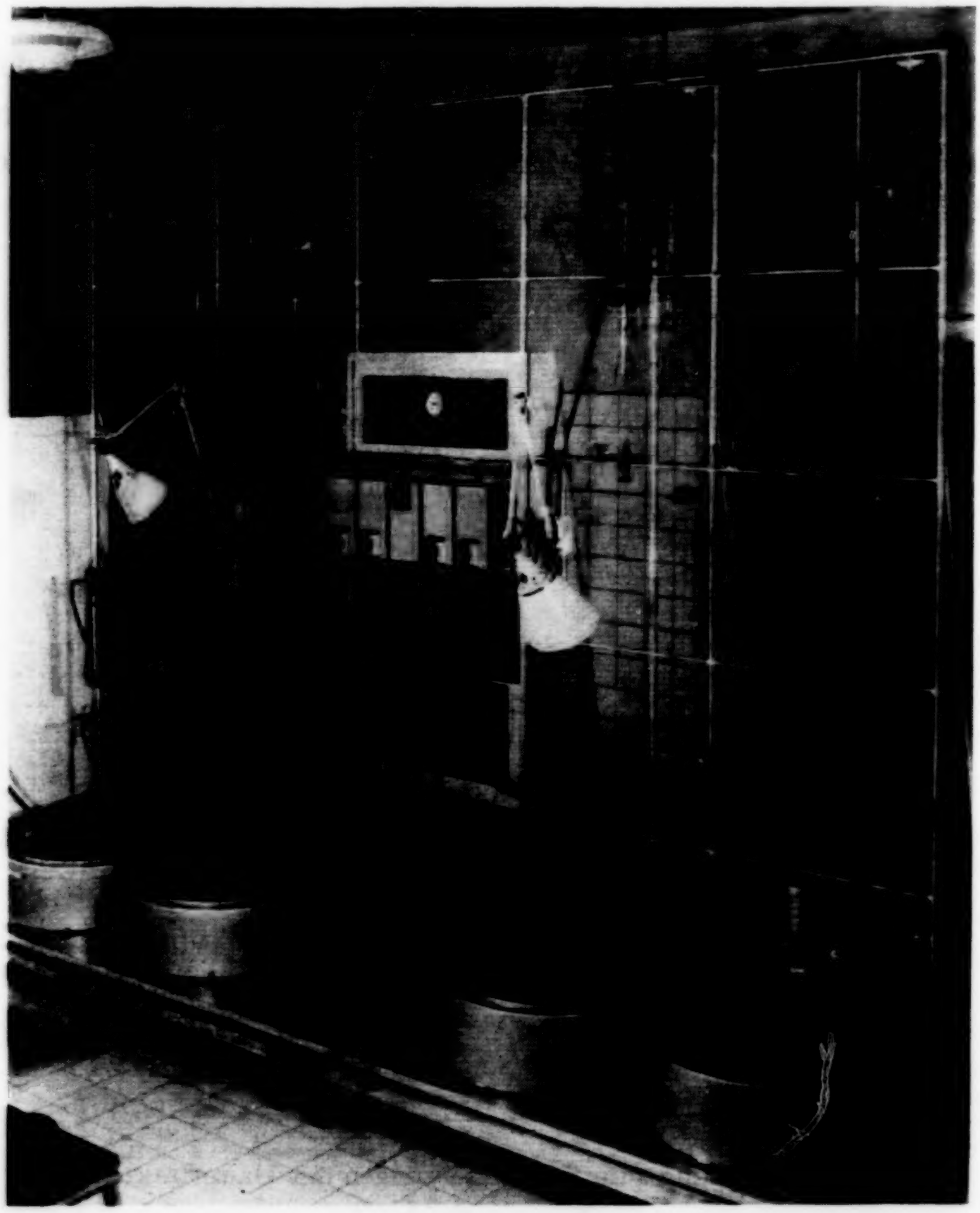

Fig. 4. Aggregat aus 4 programmgesteuerten Fraktionssammlern.

den Raum oberhalb der Paraffinölschicht. Durch kurzes Einblasen von Luft durch das Glasrohr wird Ninhydrinreagenz in die Kapillare gedrückt und tropft dann je nach Länge und Durchmesser der Kapillare in mehr oder minder grossen Zeitabständen gleichmässig ab. Durch kurzes Saugen am Glasrohr kann dieser Vorgang wieder unterbrochen werden.

Quantitative Auswertung

Anhand der Vortestung werden innerhalb eines Gipfels die Gläser der den Testfraktionen entsprechenden Hauptfraktionen zusammengefasst. Durch eine einfache Saugvorrichtung (Fig. 5) wird der Inhalt der Gläser in ein 25-ml-Messkölbchen mit Normalschliff überführt und auf 25 ml aufgefüllt; die Gesamtfraktion eines jeden Gipfels befindet sich damit in je einem Kölbchen. Genau 2 ml werden daraus entnommen und in ein 10-ml-Messkölbchen überführt; nach Zusatz von 1 ml Ninhydrinreagenz werden die Kölbchen 20 Minuten im siedenden Wasserbad erhitzt. Nach dem Auffüllen wird in üblicher Weise die Farbintensität im Photometer quantitativ bestimmt.

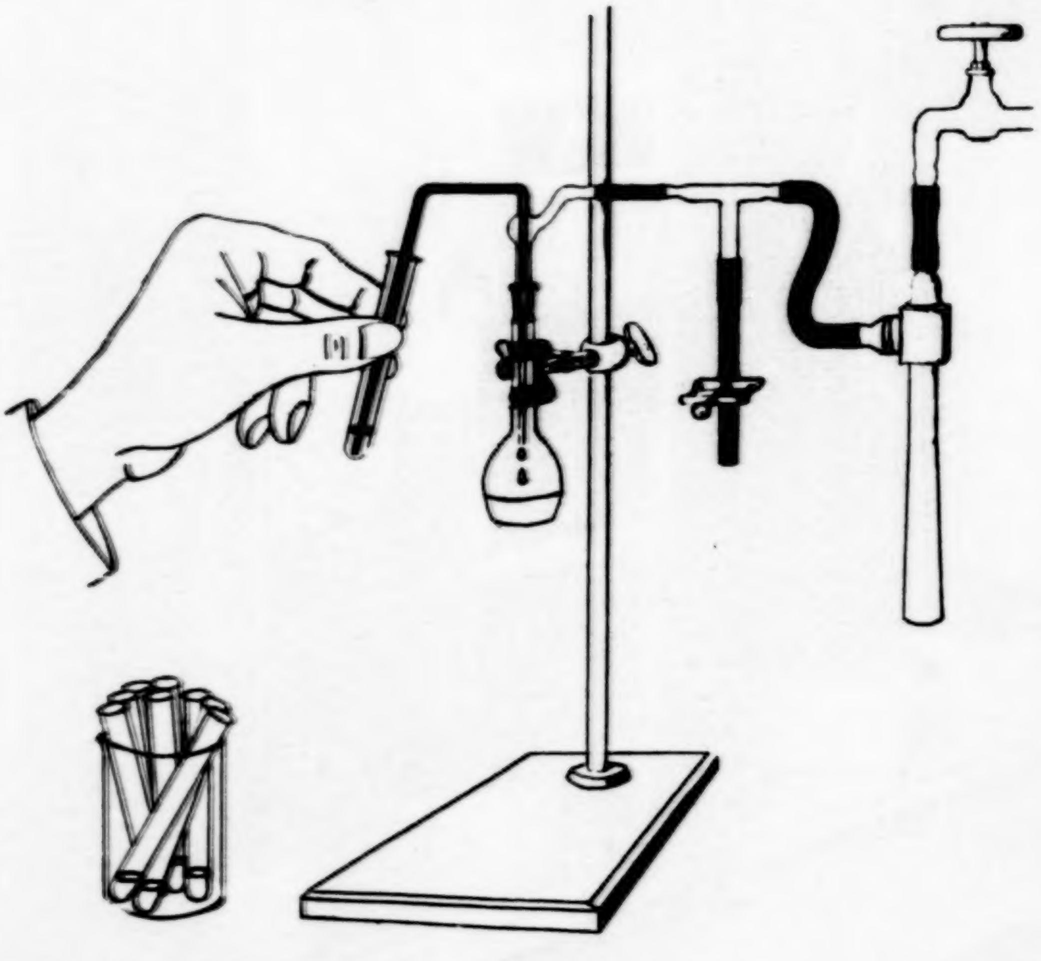

Fig. 5. Vorrichtung zum Zusammensaugen der Hauptfraktionen.

Fig. 6 zeigt eine säulenchromatographische Aminosäureanalyse, bei der jeder Strich der oberen Kurve eine quantitative colorimetrische Bestimmung bedeutet, wie sie nach der üblichen Fraktionssammlermethode erforderlich ist, während in der unteren Kurve jeder Strich die quantitative Bestimmung jeweils einer gesamten Gipfelfraktion nach dem eben beschriebenen Verfahren bedeutet, d.h. die Zahl der

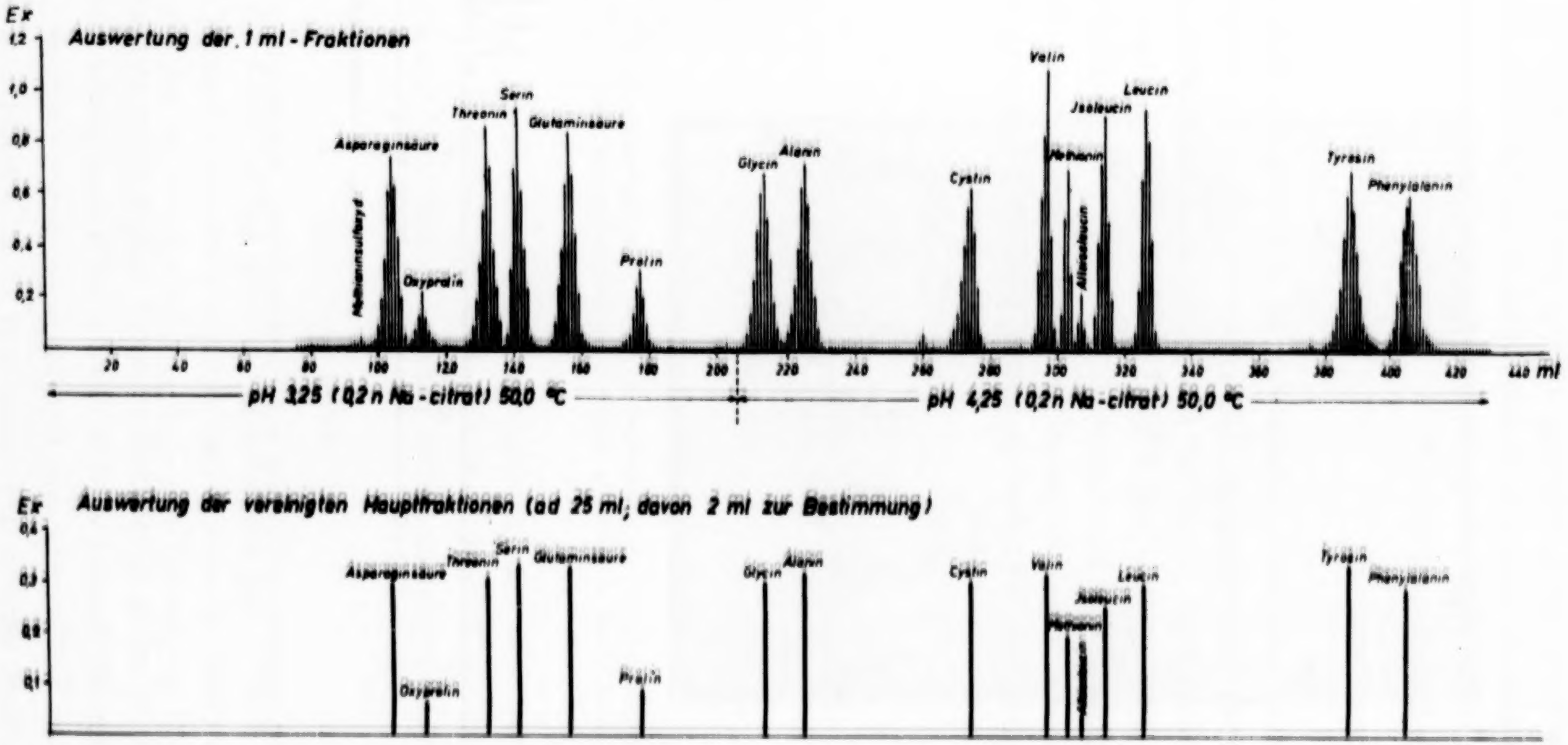

Fig. 6. Auftrennung eines Aminosäuregemisches an Amberlite CG 120, Type 3 (Partikel Diameter: 58 ± 7 μ). Säule: 166 × 0.9 cm. Druck: 0.4 atü. Durchlaufgeschwindigkeit: 15 ml/h.

quantitativen Einzelbestimmungen verringert sich bei diesem Verfahren von 300–600 auf etwa 20 zuzüglich 3 Blindwertbestimmungen. Nach der quantitativen Bestimmung stehen etwa 90% jeder 25-ml-Fraktion für Wiederholungsbestimmungen und zur qualitativen Untersuchung zur Verfügung, so dass jede Fraktion durch Vergleich mit authentischen Aminosäuren papierchromatographisch auf Identität und Einheitlichkeit geprüft werden kann. Hierzu müssen die einzelnen Fraktionen jedoch zunächst von der Pufferlösung befreit und konzentriert werden.

Entsalzung

Die Entsalzung wird an Austauschersäulen von 15 × 0.9 cm aus Dowex 50 X 4 (Na-Form), etwa 200 mesh, vorgenommen; die Glasrohre haben am oberen Ende einen Trichteransatz von etwa 7 cm Länge zur Aufnahme der Elutionsflüssigkeit.

5 ml jeder Aminosäure-Fraktion werden auf die Säule gegeben und mit 5 ml 0.01 *N* HCl nachgewaschen; anschliessend wird mit 10%igem Pyridin eluiert. Der Elutionsbereich von 4 einzeln entsalzten Aminosäuren ist aus der Zusammenstellung in Fig. 7 ersichtlich. Während die ersten 5 ml des Eluates zu verwerfen sind, werden die nächsten 20 ml in 50 ml-Bechergläsern aufgefangen und auf dem Wasserbad eingedampft; der Rückstand wird in etwa 1 ml Wasser gelöst und erneut eingedampft. Zur restlosen Entfernung von Pyridin muss dieser Vorgang 3 × wiederholt werden. Der Rückstand wird in 4 Tropfen 0.01 *N* HCl gelöst und kann dann zur papierchromatographischen Prüfung benutzt werden; die aufzutragende Menge richtet sich nach der jeweiligen Aminosäurekonzentration. Nach der Benutzung können die Säulen durch Hindurchschicken von 25 ml 0.2 *N* NaOH und anschliessend 15 ml Puffer vom pH 2.2 (0.2 *N* Na-Konzentration) regeneriert werden.

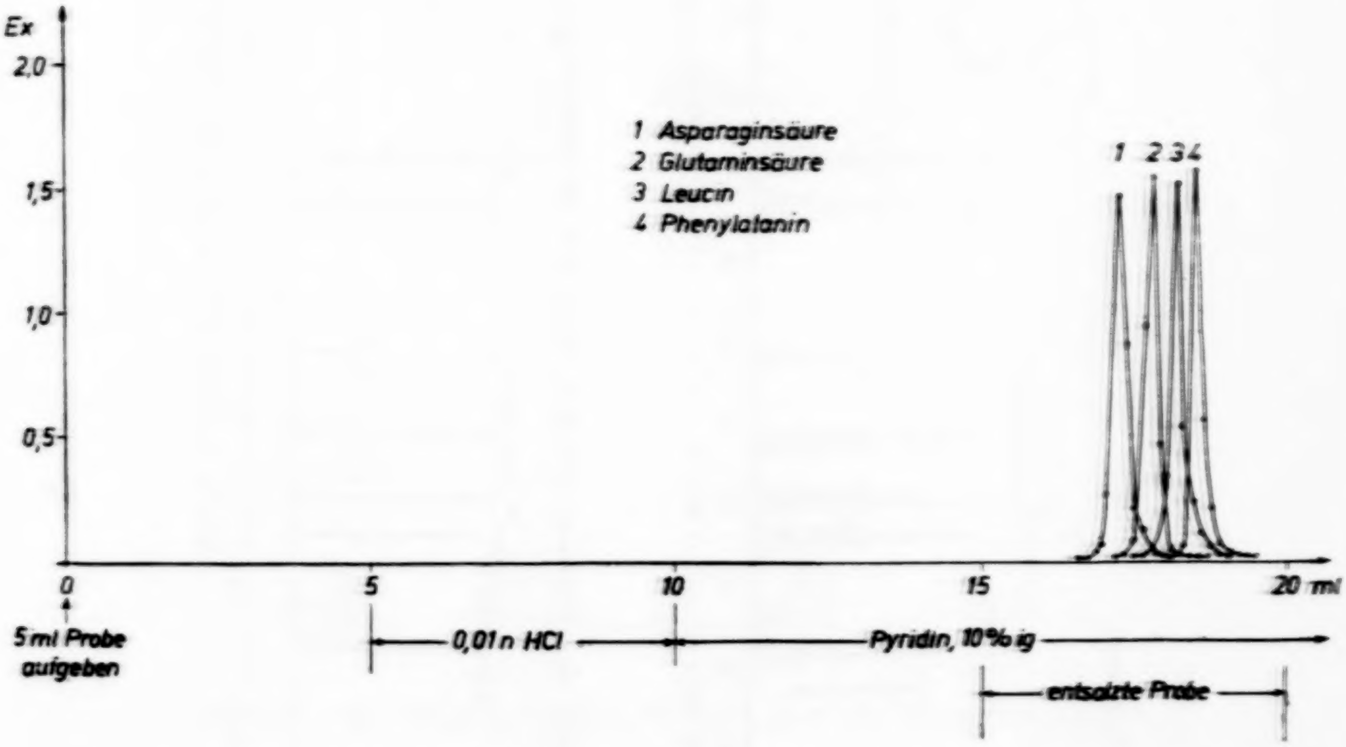

Fig. 7. Entsalzung der Aminosäurefraktion zur Identifizierung. Austauscher: Dowex 50 X 4 (Na-Form). Säule: 15 × 0.9 cm.

Papierchromatographische Untersuchung

Zur Papierchromatographie verwenden wir 6 in einem Bogen zusammenhängende Keilstreifen[7] (Schleicher & Schüll 2045b Gl mit Ausstanzungen), von denen abwechselnd je ein Streifen als Vergleichs- und ein Streifen als Testchromatogramm dient. Fig. 8 zeigt ein derartiges Chromatogramm, bei dem der Serin-, Valin- und Methioningipfel auf Identität und Einheitlichkeit geprüft werden. Auch Leucin/Isoleucin lassen sich auf diese Weise eindeutig nebeneinander nachweisen.

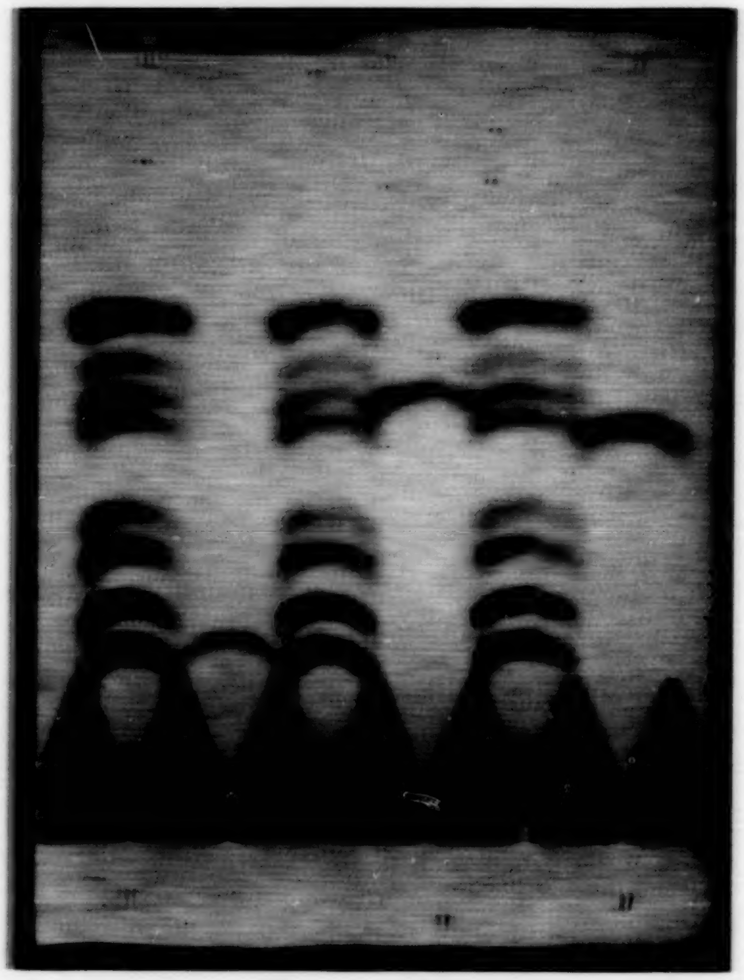

Fig. 8. Identifikationschromatogramm auf Keilstreifenbogen. Vergleichschromatogramm: 1, 3, 5. Testchromatogramm: 2 Serin, 4 Valin, 6 Methionin.

DANK

Allen, die an der Entwicklung und an dem Aufbau des Gerätes Anteil hatten, vor allem Herrn F. PANNING, der den mechanischen Teil des Gerätes ausführte, und Herrn R. GÜNTHER, der die elektrische Schaltanlage mit besonderer Sorgfalt aufbaute, sei an dieser Stelle herzlich gedankt.

ZUSAMMENFASSUNG

Es wird eine vereinfachte Technik beschrieben für die Bestimmung von Aminosäuren an Ionenaustauschersäulen mit Hilfe von Fraktionssammlern, die durch einen elektronischen Programmregler gesteuert werden. Die 1-ml-Fraktionen werden alternierend in Test- und Hauptfraktionen zerlegt. Die Testfraktionen werden halbquanti-

tativ ausgewertet; die Hauptfraktionen werden auf Grund dieser Testung im Bereich jedes Gipfels zusammengefasst, und in einem aliquoten Teil hiervon wird die quantitative Analyse durchgeführt. Die vollständige quantitative Analyse eines Proteinhydrolysates erfordert auf diese Weise nur etwa 20 quantitative Bestimmungen anstelle von 300–600. Darüber hinaus ermöglicht diese Methode Untersuchungen über die Identität und Einheitlichkeit der einzelnen Gipfel.

SUMMARY

A simplified technique is described for the determination of amino acids on ion exchange columns by using fraction collectors which are directed by an electronic program controller. The 1-ml fractions are alternatively divided into test and main fractions. The test fractions are tested semi-quantitatively; the main fractions are collected in the range of each peak, according to these results. In an aliquot part the quantitative analysis is carried out. A complete quantitative analysis of a protein hydrolysate requires only about 20 quantitative determinations instead of 300–600. Moreover, this method permits verification of the identity and homogeneity of the separated peaks.

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SOLVENT SYSTEMS FOR THE IDENTIFICATION OF NARCOTICS BY PAPER CHROMATOGRAPHY

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(Received January 15th, 1961)

A recent survey on paper chromatographic methods[1] for the identification of narcotics revealed that most of the solvent systems in use could be classified into three groups: acid, alkaline and neutral solvents. All the solvents of the first and second group and most of the neutral ones have an aqueous stationary phase and an alcoholic, particularly butanolic, mobile phase. Some authors use formamide, another polar substance, as an impregnant in combination with less polar mobile phases such as benzene, chloroform, etc.[2-4]. Chromatographic data in two isobutanol-containing solvents indicate[5,6] a satisfactory distribution of narcotics of the morphine group, and out of the 92 narcotics, 56 in the sulfate system and 68 in the phosphate system (mostly synthetic narcotics) have R_F-values between 0.7 and 1.0.

It is the object of this paper to report the results of a study of six paper chromatographic systems which have been developed to facilitate the separation and to produce a better distribution of R_F-values. The work has been limited to those narcotics which have practical value and to a few naturally occurring alkaloids.

EXPERIMENTAL

Material

Thirty-four commercially available narcotics were studied. The compounds were used without further purification.

Apparatus

Ascending chromatography was used for Systems 1, 2 and 3 as described previously[5]. For Systems 4, 5 and 6 an apparatus for descending chromatography in 30 × 30 × 61 cm glass jars was used.

Paper

Whatman No. 3 MM paper was used throughout. For Systems 1, 2 and 3, 36 × 46 cm cylinders were used with a line of application 2.5 cm from the lower end which accommodated 17 spots per sheet. Systems 4, 5 and 6 employed 23 × 46 cm strips with a line of application 7 cm from the upper end which held 9 spots per sheet.

Solutions

The narcotic (50 mg) was dissolved in methanol 10 ml (cryptopine in chloroform, narcotine in chloroform/methanol, narceine in N NaOH). This solution (10 µl = 50 µg) was applied to the salted and dried paper in Systems 1, 2 and 3 and to the untreated paper in Systems 4, 5 and 6.

System 1. Isobutanol–glacial acetic acid–water (100:10:24) on paper impregnated with KH_2PO_4 (0.5 M, pH 4.2) was used. Equilibration took place overnight (16 h), development was carried out in 8 h[5].

System 2. Isobutanol–glacial acetic acid–water (100:10:24) on paper impregnated with $(NH_4)_2SO_4$ (2%, pH 5.3) was used. The equilibration and development steps were the same as in System 1.

System 3. Butyl acetate–glacial acetic acid–water (35:10:3) on paper impregnated with KH_2PO_4 (0.5 M, pH 4.2) was used overnight. Equilibration and ascending chromatography were carried out until the solvent front reached 30 cm (4–5 h).

System 4. Propyl alcohol–water–diethylamine (1:8:1) was used as the mobile phase on paper impregnated with light paraffin (BP 1948) in hexane 4%. The spots were applied to the untreated paper which was then impregnated by a technique which avoids washing out of the applied material[7]. Descending chromatography was done after an equilibration time (30 min) until the solvent front reached 26 cm (4–5 h).

System 5. A mobile phase comprised of ammonium formate in water (10%) saturated with *sec.*-octanol was used on a stationary phase comprised of paper impregnated with *sec.*-octanol in acetone (20%). The techniques of application and impregnation are the same as those used in System 4. The equilibration time was 30 min. Descending chromatography to a solvent front of 28 cm (5 h) was carried out.

System 6. Light paraffin (BP 1948)–diethylamine (9:1) was used as a mobile phase on paper impregnated with 20% formamide in acetone. The techniques of application and impregnation are identical with those used in System 4. No equilibration time is needed. Descending chromatography was carried out overnight (16 h). The chromatograms were sprayed immediately after drying at 105° for 5 min.

Detection

Except for System 6, for detection of the narcotics, the chromatograms were dried at room temperature. The solvent front and spots detected under U.V. light of 3660 and 2537 Å were marked and then the chromatograms were sprayed with potassium iodoplatinate reagent[5].

RESULTS AND DISCUSSION

Solvent systems

Systems 1 and 2 have been described previously[5,8,9]. Mobile phases similar to System 3, were studied by THIES AND REUTHER for the separation of alkaloids and in particular papaverine and narcotine[10]. Impregnation with phosphate improves the compactness of the spots and the reproducibility of the R_F-values. This solvent has a good stability in contrast to the mobile phases of Systems 1 and 2. Chromatograms can be prepared with a solvent several weeks old. Re-use of the solvent, after replenishing the amount taken up by any previous chromatogram, had no detrimental effect on the reproducibility of R_F-values. For toxicologic and forensic case work the equilibration period can be omitted to save time.

System 4 resembles one of WALDI's solvent systems[11]. He worked with paper impregnated with petroleum of unspecified concentration. Replacing the propyl alcohol in this system by other alcohols, such as methanol, ethanol, isopropanol, isobutanol or butanol, produced a trend to lower R_F-values and slightly inferior spots. WALDI's chromatographic method proved unsuitable for the synthetic narcotics since high R_F-values were obtained. The basis of his method is the use of specific sets of R_F-values to identify a given compound, which sets are produced by systematically changing the polarity of the mobile phase by combining chloroform, cyclohexane and diethylamine in definite proportions. The stationary phase in WALDI's system is paper treated with formamide.

STEINEGGER AND OCHSNER[12] used a solvent with an aqueous phase containing formamide and sodium formate on octanol-impregnated paper for the separation of lobelia alkaloids. REICHELT[7] reported an improvement of the shape of alkaloidal spots if ammonium formate was added to the formamide used for the paper impregnation. These systems did not give either the desired R_F-spread or a good reproducibility with synthetic narcotics. It was found that 1% aqueous solution of ammonium formate on paper impregnated with *sec.*-octanol gives well shaped spots and that the R_F-values can be lowered by increasing the formate concentration. A 10% solution was chosen as the mobile phase of System 5 as giving the best spread of R_F-values. If the chromatograms are dried at room temperature and then sprayed with the potassium iodoplatinate reagent, a good "spectrum-of-colors" is obtained with various narcotics. Chromatograms from System 5 dried at elevated temperature before spraying show increased durability of the stained spots, but a loss of sensitivity, while chromatograms dried at room temperature develop a white-grayish background after spraying.

System 6 is a modification of another WALDI system[11] in which petroleum was replaced by light paraffin. Chromatograms from System 6 should be sprayed immediately after the expulsion of the adherent diethylamine vapours to prevent "afterrun" of the mobile phase, which may falsify the R_F-values.

TABLE I

System	*Dielectric constant of major component*	
	Stationary phase	*Mobile phase*
1	80	31.7
2	80	31.7
3	80	5–14
6	84	2.2–4.7
4	3–4	80
5	2.2–4.7	80

The solvents used in this study can be arranged in terms of the polarity of their stationary and mobile phases as shown in Table I[13]. The degree of polarity is indicated by the dielectric constant of the major component as given in column 2 and 3 of Table I. Systems 1, 2, 3 and 6 have strongly polar stationary phases in combination with mobile phases of decreasing polarity. On the other hand, Systems 4 and 5 belong to the "reversed phase" category which have a strongly-polar-mobile-phase in contrast to a weakly-polar-stationary-phase.

R_F-values

The R_F-values of 34 narcotics and related compounds in six paper chromatographic systems are given in Table II. The values are averages of 8–10 spots chromatographed on different sheets. The greatest deviation from the average value was ± 0.04 R_F units. Compounds have generally lower R_F-values in System 3 compared to Systems 1 and 2 due to the lower polarity of butyl acetate and the higher content of acid. In System 6, the change of solubility properties has a greater effect than the lowering of polarity of the mobile phase. The number of compounds with high or unchanged R_F-values is equal to that with the low values as compared to the classical Systems 1 and 2.

Both "reversed phase" systems lead to an improved distribution of the R_F-values of synthetic narcotics. Many of these can be separated from each other and from the morphine group. The latter show a satisfactory distribution in Systems 1, 2 and 3, and travel "en bloc" with high R_F-values in Systems 4 and 5 and with low R_F-values in System 6. Myrophine is an exception to this rule since due to its myristic acid radical, it behaves rather like a fatty acid. Although System 1 does not separate any of the synthetic narcotics any better than System 2, some members of the morphine group (*e.g.* morphine/oxymorphone, codeine/oxycodone and hydrocodone/oxycodone) show better separations in System 1. Other examples of group separations among the synthetic narcotics are indicated for the amidones, 23 to 27 in Table II which have low R_F-values in Systems 4 and 5, and high R_F-values in System 6; and for the phenylpiperidines 18 to 21 in Table II which have intermediate R_F-values in Systems 4, 5 and 6.

When choosing a solvent to separate related compounds, one should strive to find an R_F difference of at least 0.05 (ref.[14]) or better 0.08 (ref.[4]). This rule cannot be taken too literally, since separations in the lower R_F range can be achieved with smaller differences[15,16]. One hundred and ten pairs of compounds in Systems 1 and 2 can be found in Table II with an R_F difference of less than 0.08. Adding System 3 reduces this number of inseparables to fifty-three. With all six systems only seven pairs remain. In practice, some compounds with R_F-values in the higher range have to be added to this list. A total of 12 pairs should be considered as difficult to separate. Of these, four pairs include pyrahexyl along with myrophine, phenadoxone, alpha-acetylmethadol, and dipipanone. Pyrahexyl is the only narcotic which contains no nitrogen. Consequently it will not stain with the potassium iodoplatinate reagent and, being a phenol, can be identified by an azo-spray[17,18].

The best systems and the degree of separability of the remaining eight pairs are indicated in Table III.

TABLE II

R_F VALUES OF NARCOTICS IN SIX SOLVENT SYSTEMS

No.	Name of narcotic	R_F in system No.					
		1	2	3	4	5	6
1	Oxymorphone	0.21	0.10	0.06	0.93	0.80	0.08
2	Morphine	0.34	0.13	0.05	0.92	0.78	0.01
3	Oxycodone	0.34	0.22	0.16	0.92	0.81	0.16
4	Hydrocodone	0.40	0.26	0.23	0.95	0.81	0.08
5	Codeine	0.40	0.24	0.12	0.95	0.79	0.10
6	Ethylmorphine	0.66	0.45	0.33	0.94	0.77	0.18
7	Diamorphine	0.73	0.50	0.52	0.91	0.75	0.06
8	Thebaine	0.85	0.67	0.41	0.95	0.61	0.23
9	Hydromorphone	0.87	0.64	0.10	0.93	0.77	0.03
10	Benzylmorphine	0.94	0.84	0.62	0.97	0.34	0.30
11	Myrophine	0.08	0.08	1.00	0.01	0.01	0.97
12	Cryptopine*	0.50	0.34	0.53	0.00	0.55	0.10 (0.00)
13	Narceine*	0.80	0.72	0.62	0.82	0.78	0.00
14	Papaverine*	0.88	0.78	0.64	0.98	0.02	0.04
15	Narcotine*	0.88	0.78	0.72	s	0.02	0.10L
16	Cocaine	0.83	0.67	0.50	0.60L	0.71	0.67
17	Pyrahexyl	1.00	1.00	1.00	0.01	0.00	1.00
18	Anileridine	0.76	0.40	0.54	0.56	0.35	0.40
19	Pethidine	0.91	0.77	0.93	0.95	0.60	0.78
20	Alphaprodine	0.91	0.81	0.77	0.51	0.62	0.77
21	Alphameprodine	0.94	0.90	0.84	0.85	0.33	0.84
22	Ethoheptazine*	0.92	0.82	0.66	0.87	0.70	0.80
23	Normethadone	0.97	0.95	0.90	0.11	0.30	0.87
24	*l*-Dipipanone	0.90	0.98	0.41	0.00	0.07	0.98
25	Phenadoxone	0.90	0.98	0.90	0.02	0.00	0.98
26	Methadone	1.00	0.90	0.88	0.15	0.27	0.94
27	*l*-Isomethadone	1.00	0.90	0.90	0.02	0.19	0.93
28	Alpha-acetylmethadol	1.00	1.00	0.90	0.06	0.05	0.97
29	Propoxyphene	0.97	0.94	0.93	0.22	0.11	0.94
30	Diethylthiambutene	0.90	0.89	0.90	0.08	0.10	1.00
31	Levomoramide	0.97	0.95	0.87	0.0– 0.58	0.02	0.65
32	Levallorphan	0.95	0.92	0.66	0.88	0.60	0.58
33	*dl*-Methorphan	0.90	0.92	0.78	0.47L	0.36	0.91
34	Phenazocine	0.95	0.92	0.91	0.67	0.07	0.82

s = streaking; L = elongated spot; (0.00) = some material remains at start.

* Not narcotic under international law.

In conclusion it should be pointed out that it is not the intention to present a complete scheme of separation of narcotics based on R_F-values. Although such schemes have been suggested, for example for 70 alkaloids using six solvent systems and nine sprays[4], it appears unrealistic to try to do the same for narcotics. Furthermore each scheme of this nature which is designed for a limited number of compounds becomes obsolete as soon as a new member shows up. If, on the other hand, an unknown narcotic is chromatographed in six systems one obtains a chromatographic pattern, which, if it does not lead to complete identification, at least it considerably reduces the number of possibilities. The result of the chromatographic analysis will complement those of other methods like microcrystal and color tests, or U.V.- and I.R.-spectroscopy, depending on the kind and amount of material available. The chromatographic patterns of new or rare narcotics can be added to the existing collection increasing its value.

TABLE III

SEPARABILITY OF RELATED PAIRS OF NARCOTICS

Pair	*Best system*	*Separability*
Myrophine Phenadoxone	3	Overlap: blue spot of M on top of violet spot of P
Narcotine Papaverine	3	Little overlap: other systems available[8, 19–21]
Dipipanone Phenadoxone	5	Separable
Methadone Normethadone	4	Little overlap: in System 5: spot of M red-violet, spot of N violet*
Myrophine Alpha-acetylmethadol	5	Separable
Myrophine Dipipanone	5	Separable
Alpha-acetylmethadol Dipipanone	5	Overlap: brown-violet spot of D on top of red-violet spot of A
Alpha-acetylmethadol Phenadoxone	5	Separable

* See also differentiation of metabolites[22].

ACKNOWLEDGEMENTS

The authors wish to acknowledge the help of Dr. R. A. Chapman, Dr. L. I. Pugsley, and Dr. C. A. Morrell and thank them for their permission to publish this work. Thanks are due to the following organizations supplying the narcotics used in this study: Endo Lab., Richmond Hill, N.Y., E. Merck, Darmstadt, Germany, T. & H. Smith, Edinburgh, Scotland, Merck, Sharp & Dohme, Rahway, N. J., Hoffmann-La Roche, Nutley, N. J., Abbott Labs., Chicago, Ill., John Wyeth & Brothers, Canada, Eli Lilly & Co., Indianapolis, Winthrop Stearns, Windsor, Ont. and The U.S. Public Health Services, Bethesda, Maryland. Special help from Drs. Harris Isbell and Nathan B. Eddy, and Messrs. R. C. Hammond and H. I. Anslinger in obtaining samples is acknowledged.

SUMMARY

Six solvent systems, two of them of "reversed phase" type, are recommended for the paper chromatographic separation of narcotics. A chromatographic pattern obtained in these systems will facilitate the identification process.

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PAPER CHROMATOGRAPHIC SEPARATION OF MESOPORPHYRINS I AND IX

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(Received June 22nd, 1961)

INTRODUCTION

The separation of porphyrins by column or paper chromatography has been reviewed by FALK[1,2]. Under suitable conditions, porphyrins with differing numbers of carboxylic functions may be separated from each other. The separation of position isomers by paper chromatography is also possible in certain instances. Thus, in the method of CHU, GREEN AND CHU[3] coproporphyrin tetramethyl esters I and III are separable. With the system introduced by FALK AND BENSON[4], the uroporphyrin octamethyl esters are similarly separable into pairs. Isomers I and II run together as do also isomers III and IV. Using the unesterified coproporphyrins, and a 2,6-lutidine–water system, the coproporphyrin isomers I, II and III may be separated from each other, but isomers III and IV run together[5-7]. No chromatographic separation of dicarboxylic porphyrin isomers has yet been described.

Free coproporphyrins and uroporphyrins occur in small amounts in normal biological materials, the III series isomers usually predominating, but in certain pathological conditions, for example, congenital porphyria, the proportion of isomer I far exceeds that of III. Naturally occurring haemoproteins such as haemoglobin, myoglobin, catalase, peroxidase and cytochrome *b* contain as their prosthetic group a ferroprotoporphyrin which is considered, on present evidence, to be haem derived entirely from the protoporphyrin designated IX by FISCHER[8]. This isomer is theoretically derivable from aetioporphyrin III and thus belongs also to the isomeric series III. No series I type of protoporphyrin has been found in nature despite search and some false reports[9,10]. The difficulty of detecting a series I protoporphyrin admixed with protoporphyrin IX is very considerable without the aid of chromatography and would inevitably demand much material.

Protoporphyrins may be easily converted to the more stable mesoporphyrins without alteration of isomeric type and we have now devised a paper chromatographic method which separates mesoporphyrin I from mesoporphyrin IX.

EXPERIMENTAL

Materials

Synthetic dimethyl esters of mesoporphyrin I and of mesoporphyrin IX were available from the collection of Professor H. FISCHER. A specimen of mesoporphyrin IX dimethyl ester, which had been prepared by one of us (C. RIMINGTON) from the protoporphyrin of rats' faeces, was also used.

For hydrolysis, the esters are left in contact with 7 N HCl at room temperature in the dark for 42 hours. Excess acid is then removed in a vacuum desiccator containing NaOH.

Apparatus and procedure

The apparatus and general procedure are similar to those used by ERIKSEN[7]. The porphyrins (about 0.3 µg) *freshly* dissolved in pyridine (AnalaR*) are applied to Whatman No. 1 paper for chromatography and an ascending chromatogram is developed overnight with 2,6-lutidine–water (5:1) in an atmosphere of 0.88 s.g. NH_4OH and at a temperature of approximately 20°. After about 20 min in a chromatography drying oven at 50°, the porphyrin spots are located by their red fluorescence under ultra-violet light. The lower limit of detectability is about 0.06 µg and not more than 0.5 µg should be applied.

RESULTS

The R_F values found are recorded in Table I.

Mixtures were prepared of mesoporphyrins I and IX as indicated in Fig. 1, the total being 0.3 µg in each case.

TABLE I

POSITIONS OF MESOPORPHYRINS I AND IX ON PAPER CHROMATOGRAPHY

Material	R_F
Mesoporphyrin I (H. FISCHER's collection)	0.3
Mesoporphyrin IX (H. FISCHER's collection)	0.41
Mesoporphyrin IX (prepared from protoporphyrin of rats' faeces)	0.425
Mixture of mesoporphyrins I and IX	0.3 and 0.425

These were chromatographed with the results shown. 10% of isomer I was clearly distinguishable, there being two spots connected by faint fluorescence. 5% of isomer I was just detectable by tailing of fluorescence towards the I position when compared with the clear spot of pure mesoporphyrin IX.

DISCUSSION

The technique described offers the possibility of detecting, as mesoporphyrin, 5% or more of protoporphyrin I admixed with the ubiquitous isomer IX. It is still uncertain whether or not any protohaem or protoporphyrin of the I series occurs in either normal or pathological conditions.

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* High purity reagent as supplied by Hopkin & Williams, Ltd., England.

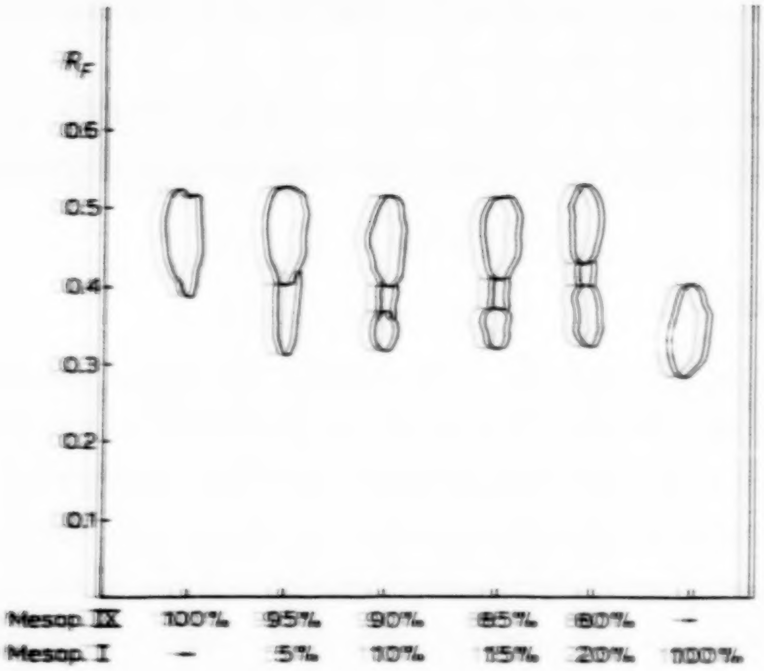

Fig. 1. Chromatographic separation of artificially prepared mixtures of mesoporphyrins I and IX. For details, see text.

The method has been applied to the investigation of a protoporphyrin present in unusually large amount in the erythrocytes of a patient suffering from a hitherto undescribed type of erythropoietic porphyria (MAGNUS, JARRETT, PRANKERD AND RIMINGTON[11]). Only protoporphyrin IX was detected.

SUMMARY

Using a 2,6-lutidine–water (5:1) system in an atmosphere of 0.88 s.g. ammonia, it is possible to separate mesoporphyrins I and IX. Five parts of the former is detectable when admixed with 95 parts of the latter. Since haems and protoporphyrins may be converted to mesoporphyrins without change in isomer type the technique renders possible the detection of this proportion of protoporphyrin I in haem or porphyrin-containing biological materials.

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ADSORPTION PAPER CHROMATOGRAPHY OF INORGANIC SUBSTANCES

VI. THE ADSORPTION PAPER CHROMATOGRAPHY OF THE COMPOUNDS OF BIVALENT TIN WITH PLATINUM METALS

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The coloured compounds obtained when solutions of platinum metals in HCl are mixed with $SnCl_2$ were first considered to be colloidal forms of the metals[1]. A series of papers by AYRES *et al.*[2] deals with their application in spectrophotometry. The nature of the compounds was also studied and for the platinum compound the formula $(PtSn_4Cl_4)^{4+}$ is suggested on the basis of the analysis of its salt with phenylarsonic acid.

Similar compounds were also observed when platinum metals were mixed with Sn(II) in HBr[3,4]. One of us[5] recently studied the paper electrophoresis of these compounds and noted that they migrate as anions in HCl (in an electric field) and that they are adsorbed on paper. It was also noted that Rh(III) when mixed with Sn(II) in HCl gives a red compound which may be converted to a yellow form by addition of HCl, by extraction with butanol or by removing the Sn(II) during electromigration. The yellow form is not identical with any of the ionic forms of Rh(III).

In this paper a detailed study of the adsorption of the Sn(II)–platinum metal compounds on cellulose is described.

EXPERIMENTAL

Whatman No. 3MM paper and ascending development on narrow strips of paper (3 cm wide) on which the solution was applied as a thin band, were employed throughout. No reagents are necessary as the colours of the compounds are very intense.

Preliminary experiments showed that it is essential to include Sn(II) in the solvent. The Pt and the Pd compound spots disappear during development with 6 *N* HCl and the red rhodium compound is separated into a yellow comet (R_F: 0.49 to 0.79) and a Sn(II) spot (R_F: 0.83).

(1) *The compounds with stannous chloride in HCl*

The following compounds of the platinum metals were employed: a solution of H_2PtCl_6 in dilute HCl, commercial $PdCl_2$, commercial $RhCl_3$, OsO_4, commercial $RuCl_3$ and K_2IrCl_6. Solutions of about 1 mg of these compounds in about 1 ml of water were mixed with equal volumes of *M* $SnCl_2$ in HCl. Fig. 1 shows the R_F values of the Pd and the Pt compounds when the concentration of HCl, of $SnCl_2$ or of H^+ (by replacing by Li^+) is varied in the developing solution. Pt and Pd always yield well defined spots and give a better separation than can be obtained by partition chromatography of the chloro-complexes. Rhodium forms an elongated spot with a red coloured front and a yellow rear. Fig. 2 shows the extent of the rhodium zone and its variation with the concentration of $SnCl_2$ and HCl. The movement of all the platinum metals in *M* $SnCl_2$–6 *N* HCl is given in Table II. Solutions that have been heated on the water-bath do in some instances change their colours. Except for Pd, which after heating forms a trail (colloidal Pd?), there is little difference in the chromatographic behaviour.

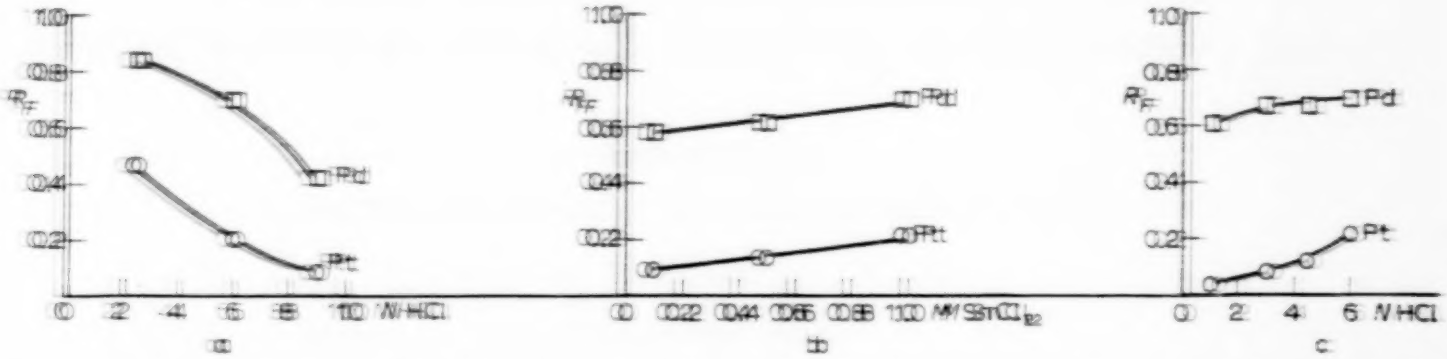

Fig. 1. (a) The variation of the R_F values of Pt and Pd with the concentration of HCl in the solvent. The concentration of $SnCl_2$ is maintained at 1 *M*. (b) The variation of the R_F values of Pd and Pt with the concentration of $SnCl_2$ in the solvent. The concentration of HCl is maintained at 6 *M*. (c) The variation of the R_F values of Pd and Pt with the concentration of hydrogen ions. All solutions contain 1 *M* $SnCl_2$ and the ratio of LiCl:HCl is varied while the total concentration of LiCl + HCl is maintained at 6 *M*.

TABLE II

R_F VALUES OF THE STANNOUS CHLORIDE COMPOUNDS OF PLATINUM METALS IN 6 *N* HCl

Paper: Whatman No. 3 MM. Solvent: 1 *M* $SnCl_2$ in 6 *N* HCl

Platinum metal	*Solution of the metal ion mixed with $SnCl_2$ in 6 N HCl*		*Solution of the metal ion mixed with $SnCl_2$ in 6 N HCl and heated a few minutes on the water-bath*	
	R_F value	*Spot colour*	*R_F value*	*Spot colour*
Pt (H_2PtCl_6)	0.21	orange	0.21	orange
Pd ($PdCl_2$ commercial)	0.70	blue	0–0.70	dark
Rh ($RhCl_3$ commercial)	0.13–0.56	yellow to red	0.13–0.56	yellow to red
Os (OsO_4)	0.69	pale brown	0.51	yellow
Ru ($RuCl_3$ commercial)	0.77	pink	0.46 (elongated)	yellow
Ir (K_2IrCl_6), two spots	0.18	yellow-brown	0.13 comet to	yellow
	0.78	yellow-brown	0.87	yellow

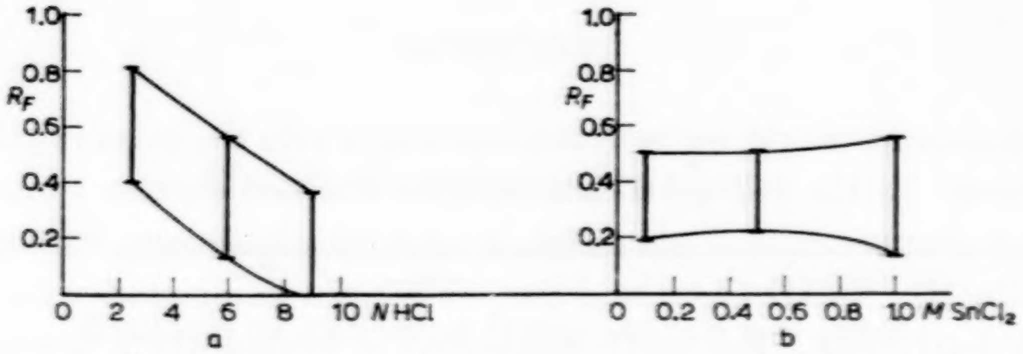

Fig. 2. (a) The variation of the height of the rhodium comet with the concentration of HCl. The $SnCl_2$ concentration is always 1 *M*. (b) The variation of the height of the rhodium comet with the concentration of $SnCl_2$. The concentration of HCl is always 6 *M*.

(2) *The compounds with stannous bromide in HBr*

We prepared a solution of $SnBr_2$ in HBr by dissolving 30 g of Sn in 250 ml conc. HBr and diluting this to 500 ml. An *M*/2 Sn(II) solution in 3 *N* HBr is so obtained, which is identical to the solvent used by PANTANI AND PICCARDI[4] for spectrophotometric studies. The R_F values and the colours of the compounds are shown in Table II. Separations of mixtures of Ru–Pd, Os–Ru, Pt–Pd, Pt–Rh–Pd were carried out successfully.

TABLE II

R_F VALUES OF THE STANNOUS BROMIDE COMPOUNDS OF PLATINUM METALS IN 3 *N* HBr

Paper: Whatman No. 3 MM. Solvent: *M*/2 Sn(II) in 3 *N* HBr (30 g of Sn dissolved in 250 ml conc. HBr and diluted to 500 ml).

Platinum metal	*R_F value of the solution of the metal ion mixed with an equal volume of the solvent*	
	R_F value	*Spot colour*
Pt (H_2PtCl_6)	0.24	red
Pd ($PdCl_2$ commercial)	0.6	dark
Rh ($RhCl_3$ commercial)	0.37	yellow
Os (OsO_4)	0.76	yellow
Ru ($RuCl_3$ commercial)	0.81	green
Ir (K_2IrCl_6 either dissolved in water or boiled with HBr) three spots	0.19	red
	0.32	yellow
	0.87	yellow
Ir, after heating the mixture of the Ir(IV) salt with $SnBr_2$–HBr for 2 or more minutes on the water-bath four spots	0.23	red
	0.37	yellow
	0.65	yellow
	0.88	yellow

The multi-spots obtained with iridium require further study. The same chromatogram was obtained irrespective of whether the chloroiridate was directly mixed with the Sn(II)–HBr solution or was first boiled with HBr. Heating was carried out for exactly two minutes, as recommended for colorimetry[3], and for longer periods. Invariably four spots were separated from heated solutions. The intensity of the spots, however, varies somewhat with the time of heating.

DISCUSSION

In this and in previous work[5] we were also concerned with the nature of the compounds formed between Sn(II) and platinum metals. The assumption that the platinum metal is zerovalent seems to be doubtful, at least for ruthenium. Partition chromatography of Ru(II), Ru(III)Cl_6^{3-} and the Sn(II)–Ru compound with butanol–3 *N* HCl showed clearly that a Ru–Sn(II) compound is formed which differs in behaviour from either Ru(II) or Ru(III). Since the potential of Ru(II)/Ru(III) is 0.08 V it seems unlikely that the Ru(III) was reduced by $SnCl_2$ (E_0: 0.14 V) and rather suggests that the Ru–Sn(II) compound would have Ru(III) as the central atom. The findings that the Pt, Pd and Rh compounds are anions would also suggest a structure similar to heteropolyacids, in which the platinum metal is the central atom and is surrounded by four (or perhaps more) $SnCl_3^-$ (or $HSnCl_4$) groups. The similarity with other heteropolyacids is again shown in their stability in acid solutions and the ready dissociation of the Sn(II) from the Rh in the case of the red rhodium compound.

SUMMARY

Some of the compounds of Sn(II) with platinum metals may be separated by adsorption chromatography on paper. Their behaviour in $SnCl_2$–HCl and $SnBr_2$–HBr was noted.

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Short Communications

Elution of proteins from starch gel into "Pevikon C-870"

In the last few years numerous techniques have been described for eluting proteins from starch gel[1-3]. The matter is also discussed by SMITHIES[4]. All methods need more or less complicated apparatus and have the undesirable feature of contaminating the proteins with large amounts of soluble starch. Chromatography of the eluate can eliminate soluble starch[5] but the procedure appears to be rather laborious.

The aim of this report is to present a method without these disadvantages. Serum proteins are separated by starch gel electrophoresis[6] and segments of gel containing the proteins to be eluted are inserted in corresponding spaces prepared in a PVK* block[7]. Correct PVK–starch gel–PVK junctions are obtained by gently pressing the PVK (lightly wetted by adding drops of buffer) against the starch gel segment. Then by performing electrophoresis the proteins migrate into the PVK from which they are easily eluted[7]. The recovery of serum proteins including albumin, transferrin and Sα_2-globulin ranges between 93 and 97%, and up to 20 gel segments can be handled simultaneously.

An important point to study was the fate of the starch eluted from the gel segment during electrophoresis. Either segments (1-2 × 1.7 × 12 cm) or larger slabs of starch gel, with or without serum proteins, were inserted in PVK beds and electrophoresis carried out up to 23 hours. The PVK blocks were cut in segments of 1 cm each and the presence of soluble starch was investigated in the eluates either by addition of iodine or, for quantitative estimation, by the anthrone method[8]. 18.6% of the eluted starch was dialysable. The peak of soluble starch was 2–4.5 cm cathodal to the gel segment in conditions in which the proteins moved 2–6 cm towards the anode. In the anodal PVK segments the reaction for starch was positive in the one in contact with the starch gel and only traces of sugars were detectable in the next two.

Thus by allowing the proteins to run in the PVK block about 5 cm ahead of the gel segments it is possible to eliminate carbohydrate contaminants completely. However, a protein fraction could become contaminated by starch from the preceding segment. This was eliminated by enveloping the gel with a cellophane sheet except on the anodal side. Subsequently only dialysable starch was found behind the gel. However, the simplest and most reliable way is obviously to insert the gel segments at great enough distances — about 5 cm — from each other.

In the experiment shown in Fig. 1 ^{131}I-labelled[9] serum proteins were used. After autoradiographic localisation three segments of starch gel were cut and inserted in a

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* PVK = Pevikon C-870.

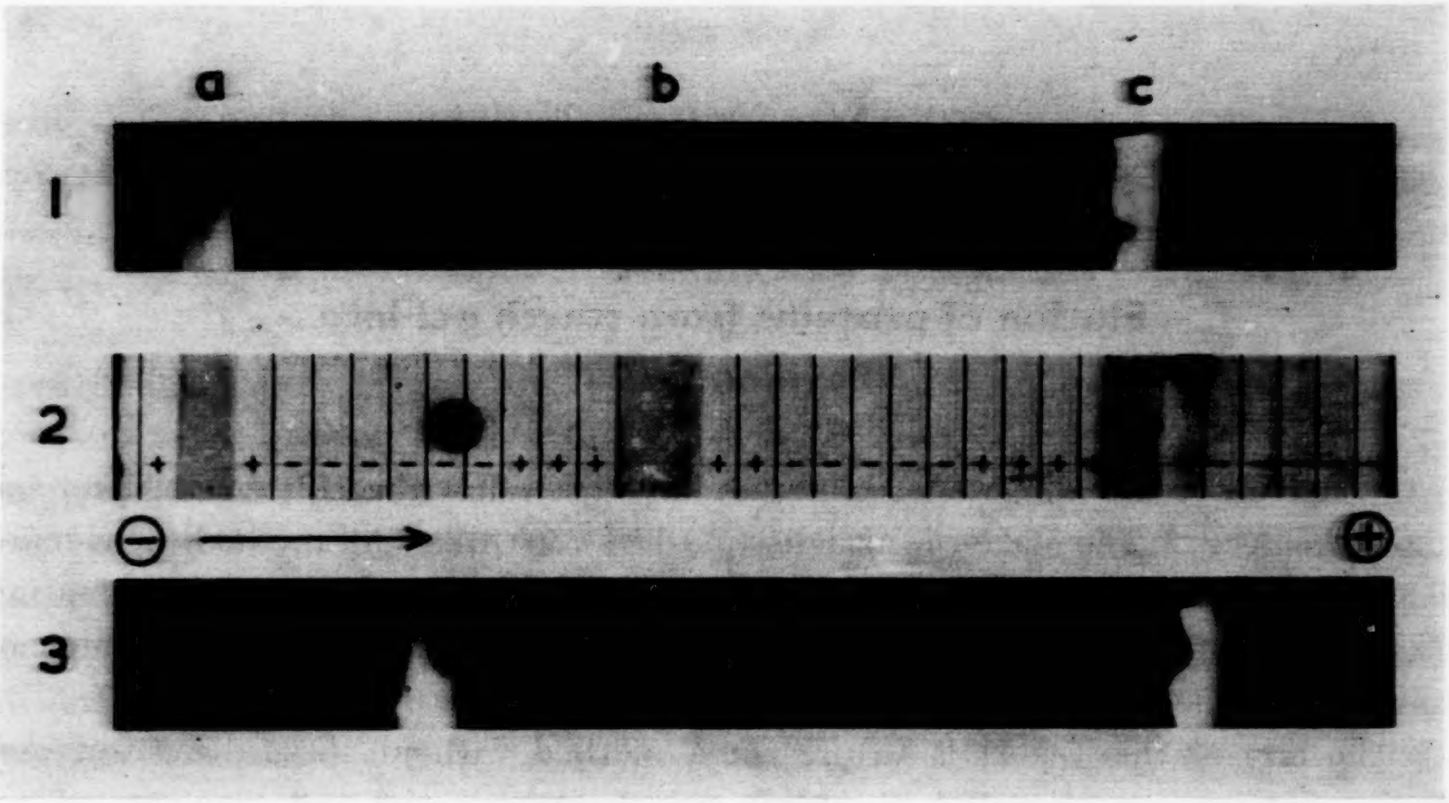

Fig. 1. (1) Autoradiography before electrophoresis: the ^{131}I proteins are in the starch gel segments. (a) albumin; (b) albumin dimer; (c) transferrin. (2) The arrangement of gel segments in the PVK bed photographed at the end of the run. The symbols + or — indicate the reaction for starch. (3) Autoradiography after electrophoresis: the ^{131}I proteins have migrated into the PVK.

PVK block (33.5 × 17 × 1.2 cm) equilibrated with borate buffer (boric acid 0.3 *M*, sodium hydroxide 0.075 *M*; pH 8.45). Electrophoresis was performed at +3°, for 10 hours employing a potential gradient of 3.5 V/cm. Radioactivities were shown to be quantitatively transferred from the starch gel into the PVK although transferrin only migrated a short distance from the gel segment.

If the segments of gel are inserted too near the anode it was noticed that migration of the proteins at a distance from the gel is prevented presumably by hydrodynamic flow.

Further studies of the purification of plasma proteins using this and other methods are in progress. I wish to acknowledge Dr. A. S. McFarlane's interest in this work.

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Received June 26th, 1961

Retention temperature in programmed temperature gas chromatography

Programmed temperature gas chromatography is now being used extensively for the separation of wide boiling-range mixtures, and several papers[1-4] have been published which deal with the relationship between retention time and column operating conditions for linear heating rates. One point which emerges from theoretical considerations[2] is that the retention temperature measured from the starting temperature is less dependent on heating rate than is retention time, provided that the ratio of fixed phase to moving phase capacity for the component is not too small (in which case the retention time is determined mainly by the dead volume of the column). This is supported by data which can be derived from published experimental results[3], but the logical step of replacing the chromatograph time axis by a temperature axis has not so far been reported.

In the present work a Moseley model 3S X-Y recorder was used and the column temperature, measured by a chromel–alumel thermocouple, was recorded on the X-axis. The normal detector signal was fed to the Y-axis, together with time interval pips derived from a synchronous motor–microswitch assembly for the simultaneous measurement of retention times. The 1/4 in. diameter columns was packed with 1/2 % Dow Corning silicone grease on 60–80 mesh glass beads, and could be cooled rapidly to room temperature by passing water through a copper coil wrapped around it. Representative results are shown in Fig. 1 for C_8–C_{14} paraffins, the retention time and temperature for each component being taken as 1.0 at the average heating rate

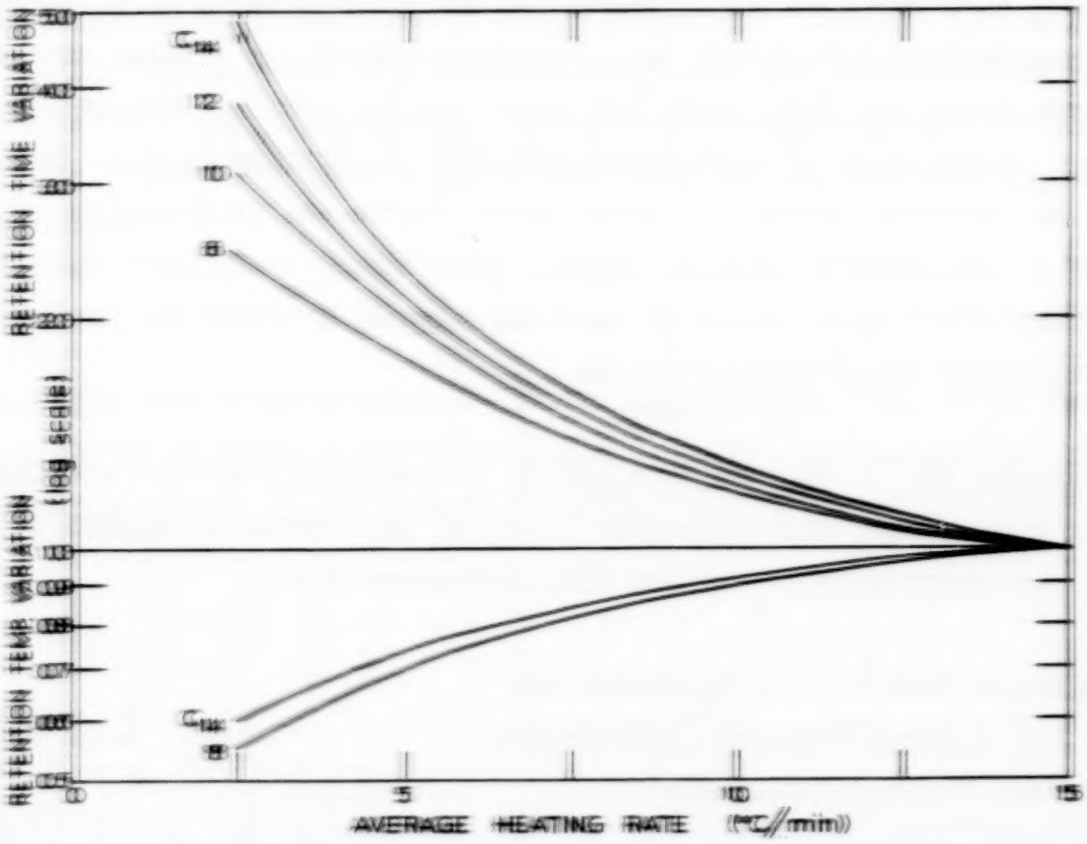

Fig. 1. Retention time (temp.) variation of C_8–C_{14} paraffins *versus* average heating rate.

of 15°C/min. Temperature changes are measured from the starting temperature of 20°C, the retention temperature variation being defined as $t_v = \frac{t_m - 20}{t_{15} - 20}$ where t_m and t_{15} are the observed retention temperatures at heating rates of m°C/min and 15°C/min respectively. Actual times (and temperatures) varied from 2.1 to 31 min (38° to 121°C). It can be seen from the figure that, even at heating rates as low as a few degrees per minute, the retention temperature is less dependent on heating rate than is retention time. Moreover Fig. 1 shows that, with change in heating rate, there is a much greater overall change in the chromatogram on a time basis than on a temperature basis. The retention temperature is also relatively insensitive to change in carrier gas velocity[2].

It is thus seen that retention intervals for characterisation of components in a sample can be measured with greater reproducibility on a temperature basis than on a time basis. Conversely, a lower degree of control is required for retention temperature measurements than for retention time measurements, and retention data are more readily interchanged between different instruments. Moreover, the results obtained with non-linearly heated columns (as in the case of Fig. 1) are more readily correlated with linear programmed temperature data. The effect of heating rate on peak shape and resolution is also more readily seen on temperature axis chromatograms than on conventional time axis chromatograms since the lengths of the latter differ so much.

In the case of a linearly heated column, the temperature axis chromatogram will be identical with the time axis chromatogram (except for a scale factor). If the detector is temperature insensitive, and responds purely on a mass per unit time basis[5], the peak areas obtained from the chart can be used to measure component concentrations on the basis of isothermal detector calibration factors. However, all detectors so far devised appear to have some degree of temperature sensitivity. In the case of a non-linearly heated column, peak areas integrated with respect to time, rather than chart areas, must be used since equal intervals on the temperature axis do not represent equal time intervals.

The author thanks Mr. I. BROWN, C.S.I.R.O. Chemical Research Laboratories, for the sample of *n*-paraffins, and Imperial Chemical Industries of Australia and New Zealand Ltd., for permission to publish this communication.

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Received July 27th, 1961

Chromatographische Trennungen von Radionukliden mittels Ammoniummolybdatophosphat auf Papierträger

Über die Ionenaustauscheigenschaften des Ammoniummolybdatophosphats (AMP) gegenüber Alkaliionen wurde mehrfach berichtet[1–9]. Trennungen von Spaltproduktgemischen an Säulenpackungen von AMP sind ebenfalls gelungen, wobei Asbest als Trägermaterial für AMP diente[10]. Vor einiger Zeit wurde die Möglichkeit einer Trennung der Alkalimetalle auf chromatographischem Wege mittels AMP-getränktem Papier beschrieben[11].

Es wurde versucht, trägerfreie Spaltproduktnuklide und trägerfreie bzw. trägerhaltige Radionuklide, die in geringer Konzentration und in kleinen Volumina vorliegen, mittels AMP, das auf Papier aufgezogen wurde (AMP-Papier), zum Zwecke der Identifizierung zu trennen.

Experimentelles

AMP wurde nach der Vorschrift von ALBERTI UND GRASSINI[11] auf Chromatographiepapier (Whatman No. 3 MM) aufgebracht. Die Beladung des Papiers betrug 9.5 mg AMP cm^{-2}. Es wurden Papierstreifen mit den Abmessungen 25 × 2 cm verwendet. Die durchschnittliche Laufstrecke betrug bei 45 min Laufzeit 12 bis 14 cm, bei 1.5 Stunden 15 bis 18 cm. Die Aktivitätsverteilung auf den getrockneten Streifen wurde mit einer Methansonde ausgemessen, wobei ein Plexiglasschieber (7 mm Dicke) mit einem Schlitz (3 mm) zwischen Streifen und Zählrohr zur Ausblendung der Strahlung verwendet wurde.

Versuche

(1) Trennungen von trägerfreiem ^{137}Cs von trägerfreiem ^{90}Sr, ^{144}Ce und ^{106}Ru bzw. Gemischen dieser Nuklide konnten mit HNO_3 (1 *M*) als Laufmittel erzielt werden. Die R_F-Werte betrugen für ^{137}Cs bzw. ^{90}Sr–^{90}Y, ^{144}Ce–^{144}Pr und ^{106}Ru–^{106}Rh 0 bzw. 1.

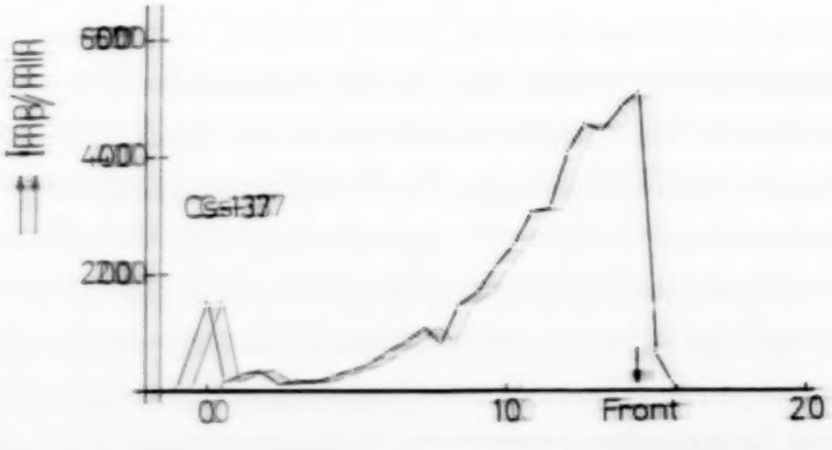

Fig. 1. Aktivitätsverteilung eines Spaltproduktgemisches (Alter: 1 Jahr) auf AMP-Papier. Laufmittel HNO_3 (1 *M*). Laufzeit 45 min, aufsteigend.

Die Abtrennung des ^{137}Cs aus einem Spaltproduktgemisch (Alter: 1 Jahr) gelang ebenfalls glatt (siehe Fig. 1). Die im Bild erkennbare Aktivitätsverschleppung wurde durch ^{106}Ru und ^{95}Zr verursacht. Mit dem Trägermaterial (Whatman No. 3 MM-Papier) lässt sich unter den gleichen Bedingungen keine Trennung durchführen, da ^{137}Cs mit der Lösungsmittelfront wandert.

(2) Ein Gemisch von trägerfreiem ^{137}Cs, ^{90}Sr–^{90}Y und ^{144}Ce–^{144}Pr konnte mit folgender Arbeitsweise getrennt werden: Mit 100 ml NH_4NO_3-Lösung (0.1 M) als Laufmittel wurde 80 min chromatographiert. Dann wurden dem Laufmittel 10 ml HNO_3 (10 M) zugesetzt und weitere 10 min chromatographiert. Die R_F-Werte waren für ^{137}Cs, ^{90}Y und ^{144}Ce 0 und für ^{90}Sr 1 für NH_4NO_3-Lösung (0.1 M). Der Zusatz von HNO_3 bewirkt jedoch, dass die offensichtlich als Hydroxyde am Startpunkt adsorbierten Nuklide ^{90}Y und ^{144}Ce–^{144}Pr mit der Front der HNO_3 wandern. Durch geeignete Wahl der Laufzeit erreicht man eine Trennung des ^{90}Y und ^{144}Ce–^{144}Pr von ^{137}Cs und ^{90}Sr.

(3) Silber und Palladium können mittels AMP-Papier ebenfalls getrennt werden. Dieses Problem stellt sich z.B. bei der Abtrennung der radioaktiven Ag-Isotope aus neutronenbestrahltem Pd. Bei Verwendung von HNO_3 (0.01 M) als Laufmittel wurde zwar eine Trennung erzielt, jedoch waren Ag- und Pd-Zone stark verwaschen (siehe Fig. 2a). Die Trennschärfe wurde durch folgende Massnahme verbessert: Die Konzentration der Salpetersäure wurde im Laufe von 1.5 h von 0.01 M auf 0.1 M verändert. Dies erfolgte in der Weise, dass nach 15, 30, 40, 50, 55, 60, 70, 75 und 80 min je 1 ml HNO_3 (10 M) zu 100 ml HNO_3 (0.01 M) (Startlösung) gegeben wurde. Dadurch wurde die Trennung wesentlich verbessert und gleichzeitig eine Konzentrierung des Pd und des Ag auf einen kleineren Fleck erreicht (siehe Fig. 2b). Die Untersuchungen mit Ag und Pd wurden mit inaktiven und aktiven Lösungen beider Elemente durchgeführt. Die Streifen wurden nach dem Versuch mit einer Lösung von Rubeanwasserstoffsäure in Eisessig zur Sichtbarmachung von Trägermengen Pd und Ag besprüht.

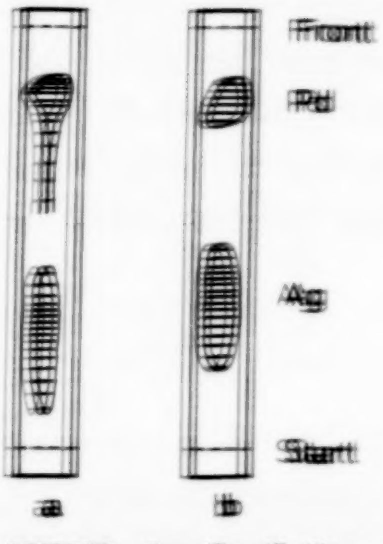

Fig. 2. Trennung von Ag und Pd auf AMP-Papier. Laufzeit 1.5 h, aufsteigend. (a) Laufmittel HNO_3 (0.01 M). (b) Laufmittel HNO_3 (0.01 M → 0.1 M).

Auf Whatman No. 3 MM-Papier konnte unter den gleichen Bedingungen keine Trennung erreicht werden, da beide Elemente mit der Lösungsmittelfront wandern.

Weitere Trennungen von Spaltprodukten und künstlich hergestellten Radionukliden werden zu gegebener Zeit mitgeteilt.

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Eingegangen den 6. Juli 1961

Chromatographie en phase gazeuse: caractérisation univoque des pics des dérivés halogénés dans un chromatogramme complexe

L'analyse des solvants complexes par chromatographie en phase gazeuse pose souvent la question de l'identification univoque des pics obtenus sur les enregistrements. Il arrive fréquemment, lorsque les solvants en question renferment à la fois des substances définies et des dérivés du pétrole par exemple, que les rares pics des premières soient plus ou moins masqués par quelques uns des nombreux pics des hydrocarbures constitutifs des seconds. C'est souvent le cas, pour certains mélanges à base de trichloréthylène, de méthylchloroforme, ou encore de chlorure de méthylène, d'une part et d'essence d'autre part, que l'on rencontre dans diverses industries. Pour ces mélanges, et, d'ailleurs, d'une manière générale, le fait qu'un pic occupe la place que devraient occuper le trichloréthylène, le méthylchloroforme ou le chlorure de méthyle, pour s'en tenir à ces trois seuls composés, ne prouve pas que l'on a réellement affaire à chacun d'eux.

Il importe donc de pouvoir attribuer sans équivoque les pics observés aux composés dont on soupçonne la présence, et, dans le cas des dérivés halogénés, une technique simple permet d'y parvenir.

Elle consiste à mettre à profit le fait bien connu que la combustion d'un tel dérivé, lorsqu'elle est accomplie en présence de cuivre métallique, s'accompagne de l'émission d'une lueur bleue verdâtre caractéristique.

On peut adapter cette réaction à la chromatographie en phase gazeuse de deux manières différentes:

(1) Si le gaz vecteur utilisé est de l'hydrogène, il suffit, à la sortie du catharomètre, de constituer un brûleur formé d'une simple aiguille hypodermique en acier inoxydable, maintenue verticale et dans le canal central de laquelle on a introduit un fil de cuivre de faible diamètre, avantageusement constitué par un brin unique détaché d'un fil électrique dit "fil souple". Le brin doit émerger de l'aiguille de 2 à 3 mm au plus.

(2) Si le gaz vecteur utilisé n'est pas de l'hydrogène, on peut mélanger l'effluent de la colonne avec de l'hydrogène, dès la sortie du catharomètre, et opérer ensuite comme précédemment.

Dans l'un et l'autre cas, on a intérêt à placer le brûleur dans une enceinte qui permet l'observation de la flamme sans être gêné par la lumière ambiante (voir Fig. 1).

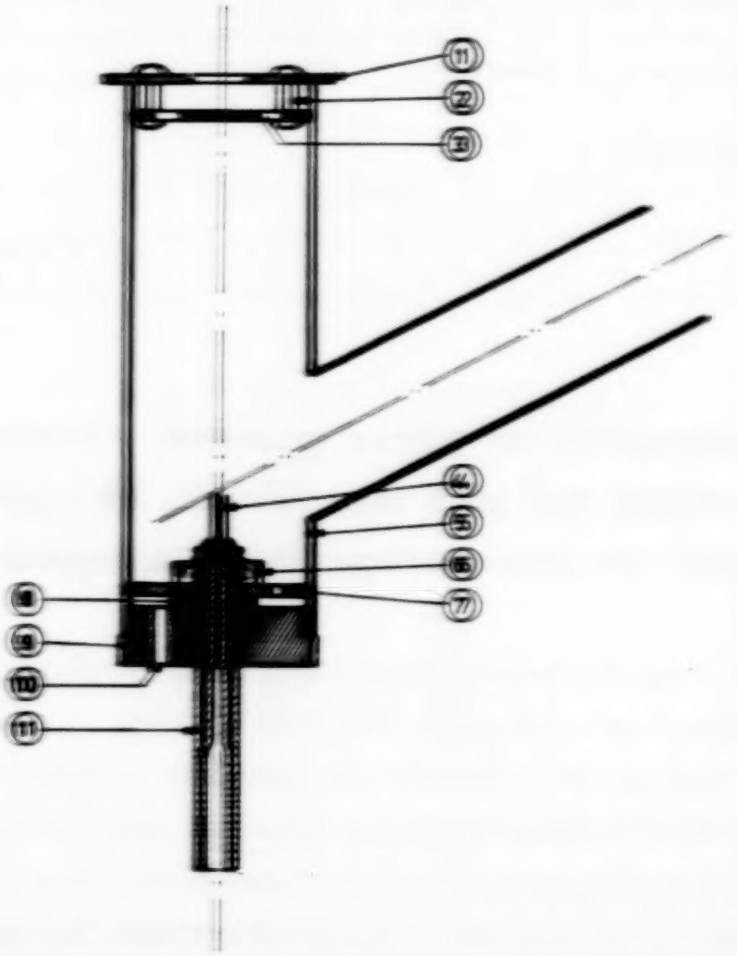

Fig. 1. Dispositif pour l'observation de la sortie des dérivés halogénés. 1 = Couvercle; 2 = Entretoises rivées; 3 = Plaque perforée; 4 = Brûleur capillaire en acier inoxydable; 5 = Enceinte permettant l'observation visuelle de la flamme; 6 = Écrou plat; 7 = Disque perforé dont les trous sont contrariés par rapport à ceux (10) du socle (9); 8 = Rondelle d'épaisseur; 9 = Socle; 10 = Arrivée d'aération; 11 = Tubulure se raccordant à la sortie du catharomètre.

Le moindre pic d'un dérivé halogéné se traduit par l'apparition de la coloration bleu-verdâtre de la flamme. Si l'on dispose d'un enregistreur grâce auquel on peut marquer un top marginal, par une commande électrique sur laquelle l'opérateur agit pendant tout le temps que dure la lueur caractéristique, on a là un moyen commode de souligner directement, sur l'enregistrement, tout pic appartenant à un dérivé halogéné quelconque, à l'exception des dérivés fluorés naturellement, quels que soient les pics qui l'entourent ou même qui interfèrent avec lui. L'identification définitive résulte de la mesure du temps de rétention du ou des pics soulignés comme à l'accoutumée (voir Fig. 2).

Une vingtaine de dérivés chlorés, bromés et iodés ont été essayés, qui tous ont donné une réponse positive à ce test simple. La réaction paraît sensible, des pics de quelques millimètres de hauteur seulement, pour un catharomètre réglé au maximum de sa sensibilité, donnant une lueur encore perceptible.

Il n'est pas impossible que l'intensité de la lueur puisse être mise à profit, au moyen d'un photomultiplicateur d'électrons, pour réaliser, sur un enregistrement séparé, le tracé des pics des seuls dérivés halogénés présents dans un mélange complexe, et peut être leur dosage.

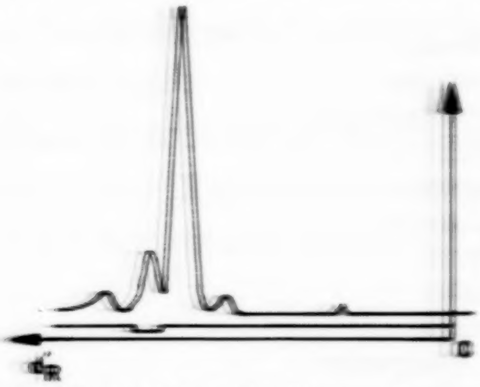

Fig. 2. Marquage du pic d'un dérivé halogéné sur le chromatogramme pendant la sortie de celui-ci.

Sans attendre ce perfectionnement, le dispositif simple décrit ci-dessus rend déjà service dans la pratique de nos déterminations analytiques courantes, pour lesquelles la caractérisation univoque des dérivés halogénés ne pose plus de problème.

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Reçu le 15 mai 1961

J. Chromatog., 6 (1961) 363–365

Dünnschichtchromatographische Trennung von Nucleinsäure-Derivaten an Celluloseschichten

Die Dünnschichtchromatographie[1] von Nucleinsäure-Derivaten an den bisher gebräuchlichen Schichten (aus Kieselgel G und Aluminiumoxid G für Dünnschichtchromatographie*) erwies sich in orientierenden Versuchen als unvorteilhaft. Der einfache Nachweis dieser Verbindungen durch Betrachtung im U.V.-Licht[2] wird durch die Eigenabsorption der Schichten gestört. Dies ist bei von uns hergestellten Cellulosepulvern für die Dünnschichtchromatographie nicht der Fall. Seit kurzem ist auch ein Cellulosepulver für dieses Verfahren im Handel erhältlich**. Das gipsfreie Cellulosepulver MN 300** ergibt ohne Bindemittel mit Aceton recht fest haftende Schichten.

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* Fa. Merck, Darmstadt (Deutschland).

** Fa. Macherey, Nagel & Co., Düren (Deutschland).

Nach unseren Erfahrungen lassen sich Nucleobasen und Nucleoside bereits mit destilliertem Wasser (Laufmittel 1) chromatographieren (Tabelle I), während für

TABELLE I

R_F-WERTE VON NUCLEOBASEN UND NUCLEOSIDEN; LAUFMITTEL 1 (s. Text)

Substanz	R_F
Adenin	0.30
Adenosin	0.53
Hypoxanthin	0.55
Inosin	0.70
Guanin	0.37
Guanosin	0.58
Uracil	0.72
Uridin	0.81
Cytidin	0.80
6-Chlorpurin	0.64
2,6-Diaminopurin	0.21

Nucleotide *n*-Butanol–Aceton–Eisessig–5% NH_3–H_2O im Volumverhältnis 4.5:1.5:1:1:2 (Laufmittel 2) geeignet ist (Tabelle II).

TABELLE II

R_F-WERTE VON NUCLEOTIDEN; LAUFMITTEL 2 (s. Text)

Substanz	R_F
Adenosin-5'-monophosphat	0.38
Adenosindiphosphat	0.26
Adenosintriphosphat	0.16
Cytidin-5'-monophosphat	0.34
Cytidindiphosphat	0.22
Cytidintriphosphat	0.13
Uridin-5'-monophosphat	0.37
Uridindiphosphat	0.25
Uridintriphosphat	0.17

Man beobachtet sehr scharf begrenzte runde Flecke. Bei Direktbeobachtung im U.V.-Licht lassen sich noch *ca.* 10^{-3} μMol Adeninverbindungen nachweisen, für Cytosin- und Uracilverbindungen liegt die Erfassungsgrenze etwas höher.

Herstellung der Schicht

10 g Cellulosepulver MN 300 (gipsfrei) werden mit 50–60 ml Aceton im Mörser verrührt und in üblicher Weise auf die Platten aufgestrichen*. Die Schichten werden 3–5 Minuten im warmen Luftstrom getrocknet und sind sofort verwendungsfähig.

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* Fa. Desaga G.m.b.H., Heidelberg (Deutschland), Grundausrüstung Nr. 600.

Ausführung der Chromatographie

Die Substanz — im allgemeinen 2–5 γ — wird 2.5 cm vom unteren Plattenrand mit einer Mikropipette aufgetragen. Der Durchmesser des Startflecks sollte 3–4 mm nicht überschreiten. Das Entwicklungsgefäss ist bis zu einer Höhe von 1 cm mit dem Laufmittel gefüllt.

Laufzeiten (Laufstrecke 10 cm): Laufmittel 1: 30 Min.; Laufmittel 2: 50–60 Min.

Die Platten werden im Trockenschrank (100°) oder im warmen Luftstrom getrocknet. Der Nachweis der Verbindungen erfolgt im Auflicht einer U.V.-Lampe (Emissionsmaximum bei etwa 260 mμ).

Als Hauptvorteile der Dünnschichtchromatographie der Nucleinsäure-Derivate an Celluloseschichten gegenüber der Papierchromatographie möchten wir die niedrigere Erfassungsgrenze und die wesentlich kürzere Laufzeit ansehen.

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[1] E. STAHL, *Chemiker-Z.*, 82 (1958) 323.
[2] E. R. HOLIDAY UND E. A. JOHNSON, *Nature*, 163 (1949) 216.

Eingegangen den 11. August 1961

J. Chromatog., 6 (1961) 365–367

Notes

Detection of nitrate on paper chromatograms with a corrosive reagent

Diphenylbenzidine in concentrated sulphuric acid is a sensitive reagent for nitrate[1]. It has not hitherto been used on paper chromatograms, since sulphuric acid solutions are too viscous and corrosive to be used as sprays or dip-reagents. The following procedure permits the use of this reagent on paper.

A small amount of reagent (0.02% diphenylbenzidine in concentrated sulphuric acid) is poured into the lower half of a petri dish, and spread in a thin film over the bottom. A piece of paper is laid on the chromatogram over the suspected position of the nitrate spot and the overlaid paper and test-piece are cut out together so that a facsimile of the test-piece is produced, together with the test-piece itself. The test-piece is laid carefully into the reagent-wetted petri dish, so that no bubbles are trapped beneath, and the lid is put on. The whole assembly is quickly inverted,

so that the test-piece is left adhering to the (now) uppermost inner surface of the dish. The nitrate, if present, will be shown by a dark blue spot. The position of the spot can be reproduced by free-hand drawing or tracing on the facsimile, which can then be fitted into the original chromatogram and fixed there with cellulose tape. This is made easier if the test-piece and facsimile are cut out with pinking shears.

The limit of detection of this procedure is 1 μg potassium nitrate, when applied to Whatman No. 1 paper in 5 μl water and developed with pyridine–ethanol–water–concentrated ammonium hydroxide (60:20:16:4). The reagent is more sensitive and specific than brucine[1], Laurent's acid-ammoniacal silver nitrate-fluorescein reagent[2], aniline-glucose reagent[3], or alkaline 2% [illegible]. These reagents give no reaction with 10 μg potassium nitrate.

The procedure could be adapted to the examination of thin-layer chromatograms by the use of glass strips or sheets in place of petri dishes. It could also be adapted to the use of other corrosive reagents which could not be used on paper in the normal way.

This work was supported jointly by the Australian Mining Industry Research Association, the Commonwealth Bureau of Mineral Resources, and the C.S.I.R.O.

Commonwealth Scientific and Industrial
Research Organisation,
Division of Plant Industry,
Black Mountain, Canberra, A.C.T. (Australia)

R. W. [illegible]

[1] F. Feigl, *Spot Tests in Inorganic Analysis*, 5th Ed., Elsevier, Amsterdam, 1958, p. 327.
[2] I. I. M. Elbeih and M. A. Abou-Elnaga, *Anal. Chim. Acta*, 23 (1960) 30.
[3] H. Wollenweber, *Naturwiss.*, 44 (1957) 38.

Received May 4th, 1961

J. Chromatog., 7 (1962) 367–368

Über die Reinigung von Urease durch Chromatographie

Bei der chromatographischen Reinigung von Trypsin hat sich CAM-Pulver (Schleicher und Schüll) als Säulenfüllung bewährt[1]. Weiterhin ist versucht worden, handelsübliche Urease-Präparate (Merck, Darmstadt) zu reinigen bzw. die Fermentaktivität anzureichern. Hierfür zeigte sich ebenfalls CAM-Pulver* als am besten geeignet. Als brauchbares Elutionsmittel erwies sich eine 0.2 N Na-Phosphatlösung.

Zur Vorbehandlung der stationären Phase ist das CAM-Pulver nacheinander mit 0.2 N HCl, aqua dest., 0.2 N NaOH und wieder mit aqua dest. gewaschen worden. Hierbei kamen auf je 10 g CAM-Pulver 100 ml 0.2 N HCl und 100 ml 0.2 N NaOH. Es ist bis zur Cl-Freiheit bzw. zur Neutralität mit aqua dest. gespült worden. Die nach der NaOH-Behandlung neutral gewaschenen Pulver konnten bei 4° bis zur weiteren

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* CAM-Pulver wurde uns freundlicherweise von der Firma Schleicher und Schüll, Dassel Kr. Einbeck, zur Verfügung gestellt.

Verwendung aufbewahrt werden. Vor dem Einfüllen in die Säulen sind entsprechende Mengen an CAM mit der genannten Pufferlösung solange vorbehandelt worden, bis pH 6.8 erreicht wurde.

Eine Säulenlänge von 35 cm hat nicht ausgereicht, alle vorhandenen Komponenten, vor allem die Fraktion mit Urease-Aktivität, von anderen ninhydrin-positiven Material abzutrennen. Demzufolge ist die Länge der Kolonnen auf 50 cm erweitert worden.

In einem typischen Versuch wurde eine 50 × 1 cm CAM-Säule mit der genannten Pufferlösung auf pH 6.8 eingestellt und mit *ca.* 40 mg Urease beschickt. Die Ureaseprobe wurde in der Pufferlösung pH 6.8 aufgelöst. Die Chromatographie erfolgte bei Raumtemperatur. Die Durchlaufgeschwindigkeit betrug 5 ml/h.

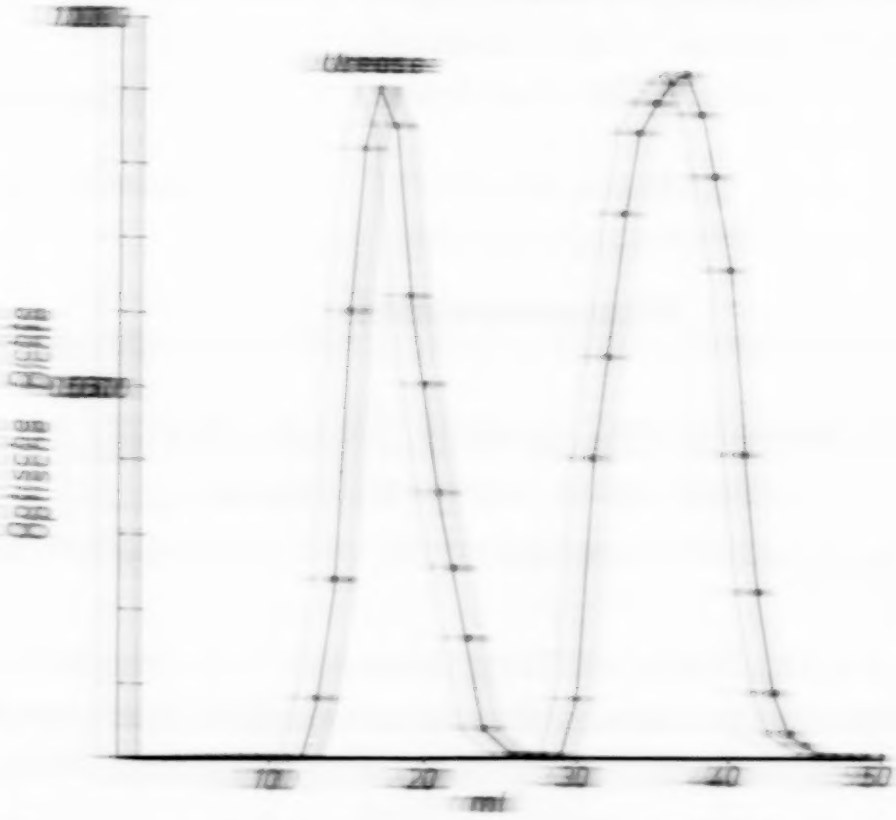

Fig. 1. Elutionsdiagramm von Urease (Merck) an 50 cm CAM-Säulen. Puffer: 0.2 M Na-Phosphatlösung, pH 6.8.

Das Eluat der Säulen wurde mit Hilfe eines Fraktionssammlers in 1 ml-Portionen aufgefangen. Von jeder 1 ml-Fraktion sind 0.5 ml zur Ninhydrin-Reaktion[2] und 0.5 ml zur Feststellung der Urease-Aktivität verwendet worden.

Die Fermentaktivität wurde in folgender Weise bestimmt. Zu jeder 0.5 ml Portion einer 1 ml Eluatfraktion sind 0.5 ml 1 *M* Harnstofflösung zugesetzt worden. Diese Proben wurden 1 Stunde lang bei 37° gehalten und anschliessend nesslerisiert.

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[1] G. KRAMPITZ UND F. KNAPPEN, *J. Chromatog.*, 5 (1961) 174.
[2] S. MOORE UND W. H. STEIN, *J. Biol. Chem.*, 192 (1951) 663.
[3] R. MÜLLER UND G. KRAMPITZ, *Z. Tierphysiol. Tierernährung u. Futtermittelkunde*, 11 (1956) 27.

Eingegangen den 15. Mai 1961

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* Direktor: Prof. Dr. H. SCHERMANN.

BOOK REVIEW

Woelm-Mitteilungen: AL 5, Aluminiumoxide WOELM zur Chromatographie, Ausgewählte Literatur, M. Woelm, Eschwege, 1961, 32 pages.

M. Woelm (Eschwege) have been for some time the manufacturers of a special brand of alumina for chromatography. The present pamphlet is a selection of abstracts of papers concerning the use of this brand in chromatography.

In each case the names of the authors, their addresses, the complete reference and an abstract of about 10 lines describing specifically the application for the alumina are given. The booklet comprises forty-seven papers and an author and a subject index.

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Announcements

FOUNDING OF THE "SOCIETÀ ITALIANA PER LO STUDIO DELLE SOSTANZE GRASSE"

(THE ITALIAN SOCIETY FOR THE STUDY OF FATTY MATERIALS)

The "Italian Society for the Study of Fatty Materials" was founded and registered on December 10th, 1960, but the news of its existence had, in fact, already been made known; the Society is established in Milan, Via Lauro, 3. It is directed by the following Officers:

President	Prof. R. RIGAMONTI
Vice-President	Dr. Prof. A. PALENI
Members of the Board	Prof. A. FABRIS
	Dr. Prof. A. MONTEFREDINE
	T. N. PLEBANI, Ing.
Secretary	Dr. Prof. G. JACINI

The aims of the Society are purely scientific and cultural, with no thought of pecuniary benefit. The essential purpose is to advance and disclose scientific and technical knowledge regarding fatty materials, as well as to promote meetings and contacts between scientists and technologists interested in all aspects of fatty materials (chemical, biochemical, medical, engineering, bromatologic, cosmetic, etc.).

The Society's activities and programmes will be published in the monthly magazine edited by the "Stazione Sperimentale Oli e Grassi" (Experimental Station for Fats and Oils), which, in agreement with the Society's objectives, will have a new title starting with the January 1961 issue, namely: "*La rivista italiana delle sostanze grasse*".

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The admission and annual fees have been fixed by the Officers as follows:

Fee for enrolment in the Society		
	(Collective members, individual members and aggregate members)	L. 5,000
Annual fee 1961		
Collective members		L. 25,000
Individual members	(including the subscription for 1961 to the periodical *La rivista italiana delle sostanze grasse*)	L. 6,000
		L. 6,000
	(without subscription)	L. 3,000
Aggregate members	(with subscription)	L. 4,000
	(without subscription)	L. 1,500

Ordinary members only are entitled to vote and can become members of the Board of Directors.

Interested persons may apply for information to the Secretarial Office of the Society in Milan, Via Lanzone 33.

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THE NOMENCLATURE OF GAS CHROMATOGRAPHY

Following the publication of papers on the nomenclature of gas chromatography by AMBROSE, KEULEMANS AND PURNELL[1], and by JOHNSON AND STROSS[2], a special Group was formed under the auspices of the Section of Analytical Chemistry of the International Union of Pure and Applied Chemistry to draw up Recommendations for a standard terminology. The Group consisted of Dr. D. AMBROSE, Chairman (Great Britain), Dr. A. T. JAMES (Great Britain), Professor A. I. M. KEULEMANS (The Netherlands), Dr. E. KOVÁTS (Switzerland), Dr. H. RÖCK (Germany), Dr. C. ROUIT (France) and Dr. F. H. STROSS (U.S.A.). The Preliminary Recommendations made by the Group were approved by the Committee of the Analytical Section in 1959 and have since been published[3]. This year Dr. RÖCK has resigned from the Group and his place has been taken by Dr. E. BAYER (Germany).

As a result of comments which have been made about the Preliminary Recommendations it is proposed that amendments should be submitted to I.U.P.A.C. on the following two points:

(1) In the section on Apparatus Performance, an equation (equation 12) is given which relates the resolution obtained to the number of theoretical plates in a column.

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This equation is only valid when the gas hold-up is negligible compared with the retention volume, a condition which is not fulfilled with capillary columns, and the equation should be deleted.

(2) In the section on retention parameters it is thought that the application of the correction factor j should be made more explicit by insertion of the following paragraph.

The correction factor j should strictly be applied only to parameters which relate to the column alone and are unaffected by the volumes of the injector and detector. The retention volume:

$$V'_R + \frac{V_G}{j}$$

(where V_G is the interstitial volume of the column) referring to an ideal chromatographic apparatus, in which the volumes of the injector and detector are zero, may be called the theoretical retention volume. For most purposes there is no need to evaluate the theoretical retention volume but the definition is included here in case the distinction is needed for didactic or theoretical purposes.

Members of the Group will be glad to receive opinions on these or any other matters for incorporation in revised Recommendations. Please write to any member or the Chairman, Dr. D. AMBROSE, National Chemical Laboratory, Teddington, Middlesex, England.

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[1] D. AMBROSE, A. I. M. KEULEMANS AND J. H. PURNELL, *Anal. Chem.*, 30 (1958) 1582.
[2] H. W. JOHNSON AND F. H. STROSS, *Anal. Chem.*, 30 (1958) 1586.
[3] *Pure and Appl. Chem.*, 1 (1960) 177; *Gas Chromatography 1960* (Editor: R. P. W. SCOTT), Butterworths, London, 1960, p. 423.

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REVUE D'ENSEMBLE

TECHNIQUES ET APPLICATIONS DE LA RADIOCHROMATOGRAPHIE EN PHASE GAZEUSE

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(Reçu le 13 juillet 1961)

I. INTRODUCTION

La chromatographie en phase gazeuse, dont le succès dans de nombreux domaines n'est plus à démontrer, n'a pas tardé à devenir une technique radioisotopique très appréciée, tant pour la séparation et la purification de molécules marquées par des isotopes radioactifs, que pour l'étude de problèmes fondamentaux en chimie des radiations et en chimie des atomes chauds. C'est en particulier dans ce dernier domaine que cette méthode d'analyse a eu les succès les plus éclatants. Commentant les progrès récents de la chimie des atomes de recul, **Harbottle**[1] souligne le rôle essentiel de la chromatographie en phase gazeuse dont l'utilisation est de plus en plus répandue:

"...Cette technique extrêmement féconde permet la séparation complète et rapide ainsi que l'identification, de nombreuses espèces moléculaires et même de molécules isotopiques telles que HT ou DT; elle a accéléré le progrès des recherches dans ce domaine tout en diminuant dans une très large mesure la durée des analyses chimiques. Enfin, elle a attiré l'attention des chercheurs sur des réactions secondaires induites par des rayonnements...". C'est ainsi que **Evans et Willard**[2] ont pu séparer après irradiation du bromure de *n*-propyle plus de vingt types de molécules marquées par ^{82}Br, alors que les premières analyses de **Libby** *et al.*[3,4] et **Chien et Willard**[5] par distillation fractionnée, après addition d'entraîneurs, n'avaient mis en évidence qu'une dizaine de ces composés[6].

2. TECHNIQUES DE LA RADIOCHROMATOGRAPHIE EN PHASE GAZEUSE*

La technique radiochromatographique se distingue de la technique ordinaire uniquement par le détecteur qui enregistre la radioactivité des solutés se présentant à la sortie de la colonne. Ces détecteurs de radioactivité ont une sensibilité supérieure à celle des détecteurs chromatographiques les plus sensibles basés sur les phénomènes

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* Quelques-unes de ces techniques ont été discutées récemment dans ce journal par **Dobbs**[23] et **James et Piper**[32].

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

(b) *Détection continue*

Les détecteurs de radioactivité placés en série avec un détecteur chromatographique tel que le catharomètre permettent d'effectuer simultanément une analyse chromatographique et radiochromatographique. L'avantage de cette double analyse est considérable: le catharomètre répond aux solutés présents en quantités pondérables marqués ou non, et le détecteur de radioactivité n'enregistre que les espèces moléculaires marquées. Un soluté présent en quantité pondérable et marqué par un isotope radioactif est enregistré simultanément par les deux types de détecteurs. Le rapport des hauteurs ou des aires des pics enregistrés par ces deux détecteurs indique après étalonnage l'activité spécifique de ce soluté. En présence d'impuretés radioactives le pic mesurant la radioactivité s'élargit et devient asymétrique. On dispose ainsi d'un excellent critère de pureté radiochimique[illegible].

1. *Détecteurs intégraux*

C'est à ce type que correspondent les détecteurs décrits par [illegible] et de façon plus détaillée par POPJAK[illegible]. Le mélange du gaz vecteur et des solutés barbote à la sortie de la colonne dans une solution scintillante (diphényloxazole en solution dans le benzène ou le xylène). En raison de leur solubilité et de leur point d'ébullition élevé, les vapeurs organiques sont retenues par la solution scintillante et la radioactivité est mesurée à l'aide d'un photomultiplicateur relié à un intégrateur pourvu d'un commutateur automatique de sensibilité et suivi d'un enregistreur à trois voies: l'une des voies enregistre la réponse du détecteur chromatographique (balance à densité gazeuse) et les deux autres voies enregistrent la radioactivité avec un rapport de sensibilité de 10 à 3. Les courbes obtenues sont des courbes en gradins caractéristiques des détecteurs intégraux. La réponse du détecteur de radioactivité est proportionnelle à la quantité de radioéléments introduite, jusqu'à 30000 impulsions par seconde. Le dispositif de POPJAK est utilisé pour l'analyse chromatographique d'échantillons radioactifs synthétisés par voie biologique. Le ^{14}C est compté avec une efficacité de 50 %, le tritium avec une efficacité de 10 %.

JAMES ET PIPER[illegible] ont fait remarquer que pour conserver une bonne sensibilité il était nécessaire de remplacer la solution scintillante lorsque celle-ci a dissous un certain nombre de solutés radioactifs.

La méthode de KARMEN ET TRITCH déjà signalée[illegible] peut être utilisée également pour la détection continue: les gaz sont retenus dans une colonne d'anthracène placée entre deux photomultiplicateurs.

Un autre dispositif de détecteur intégral a été mis au point par [illegible]. Les gaz sont condensés dans une cellule refroidie par l'azote liquide. L'une des parois de cette cellule a une épaisseur suffisamment faible pour permettre le passage du rayonnement émis par le ^{14}C, décelé à l'aide d'un compteur à fenêtre mince placé en regard de cette paroi.

2. Détection par compteurs Geiger–Müller et proportionnels

On peut, de manière très simple, faire passer le gaz sortant de la colonne devant la fenêtre d'un compteur Geiger. Cette technique radiochromatographique a été utilisée dès 1955 par Kokes *et al.*[15]. Elle a trouvé un certain nombre d'applications et peut être améliorée par l'emploi de deux compteurs à fenêtre mince entre lesquels circule le gaz ([illegible] *et al.*[16]).

[illegible][17] rappelle qu'il existe trois principes radiochromatographiques: marquage des solutés avant séparation chromatographique, irradiation neutronique des solutés après chromatographie suivie de la mesure de l'activité induite, traitement des solutés après chromatographie à l'aide d'un réactif marqué. L'auteur décrit une méthode basée sur ce dernier principe: les gaz sortant de la colonne sont brûlés en présence d'oxyde de cuivre; le gaz carbonique formé traverse un tube contenant du carbonate de sodium marqué au ^{14}C ($^{14}CO_3Na_2$) chauffé à 200°. Après échange isotopique, le gaz carbonique marqué est détecté à l'aide d'un compteur à fenêtre mince. La sensibilité de cette méthode serait 10^3 fois supérieure à la sensibilité de la cellule de conductivité thermique.

Cependant l'efficacité de comptage du ^{14}C reste toujours faible et le tritium ne peut être détecté avec ces dispositifs.

Cette efficacité augmente énormément si les gaz traversent directement le compteur. Il n'y a plus aucune paroi entre le gaz et le détecteur. WOLFGANG ET MACKAY[18] ont décrit des compteurs proportionnels à circulation de gaz et plus particulièrement un compteur destiné à l'enregistrement continu de la radioactivité d'un gaz à la sortie d'une colonne chromatographique (WOLFGANG ET ROWLAND[19]).

Les compteurs ne pouvant fonctionner avec le gaz vecteur, il est nécessaire d'introduire un gaz supplémentaire, en général le méthane, injecté dans le circuit entre le détecteur chromatographique et le compteur. Les auteurs ont étudié en détail le fonctionnement de ces compteurs et ont donné une expression du taux de comptage moyen en fonction du volume sensible du compteur et des débits du gaz vecteur et du méthane. Ces détecteurs conviennent parfaitement pour la mesure des rayonnements du ^{14}C et du tritium avec une sensibilité de 10^{-4} à 10^{-5} µC. Ils présentent sur les chambres d'ionisation les avantages d'une sensibilité 20 à 25 fois plus grande, d'une réponse plus rapide, de meilleures caractéristiques de température (le fonctionnement est encore normal à 200°). Il est enfin possible d'effectuer simultanément un enregistrement différentiel et intégral par comptage à l'aide d'une échelle du nombre total d'impulsions dues aux solutés élués.

Ce type de compteur a été utilisé par [illegible] ET TOMLINSON[20] pour la détection avec une efficacité de près de 100% du rayonnement du ^{85}Kr; ils ont confirmé que le fonctionnement était compatible avec une température de 200°, le seul effet de température étant une diminution de la longueur du palier.

Certains solutés (composés nitrés ou halogénés) peuvent "empoisonner" le compteur, perturbant son fonctionnement. On enregistre alors des pics "négatifs" dus à un décalage de la ligne de base. [illegible] *et al.*[21] ont su résoudre cette difficulté en ajoutant de façon délibérée une quantité constante de ce poison au gaz injecté dans le compteur (par exemple 1% de vapeurs de nitrobenzène).

James et Piper[13] utilisent le même type de détecteur avec une variante: le gaz vecteur est de l'argon, et les gaz à la sortie de la colonne sont brûlés en présence d'oxyde de cuivre. La vapeur d'eau est éliminée par passage sur du perchlorate de magnésium et le gaz carbonique (dont la concentration en volume est portée à 5 % par injection de gaz carbonique inactif) traverse le compteur. La méthode peut convenir à l'analyse de composés tritiés, le perchlorate de magnésium étant remplacé par du carbure de calcium.

3. *Utilisation de détecteurs à scintillations*

Les cristaux scintillateurs d'iodure de sodium activé au thallium, NaI(Tl), sont utilisés pour la détection continue de solutés marqués par des isotopes émetteurs d'un rayonnement γ. **Evans et Willard**[2] font circuler les gaz à travers un tube de verre placé dans le puits d'un cristal, et détectent 10^{-13} g de $CH_3{}^{82}Br$ et 10^{-15} g de $CH_3{}^{80}Br$ (voir aussi[21]).

Moussebois et Duyckaerts[22] opèrent de manière analogue pour détecter des iodures organiques marqués par l'^{131}I.

Herr *et al.*[23] font circuler le gaz à travers une spirale de verre de volume interne 10 ml, placée sur un cristal NaI(Tl) de 2.5 pouces et détectent avec une bonne efficacité des bromures organiques marqués par le ^{82}Br. L'utilisation d'un cristal NaI(Tl) "tunnel" traversé directement par la colonne chromatographique donne également d'excellents résultats[24].

Dans tous ces dispositifs, la sensibilité est fonction de la durée de séjour du gaz radioactif dans le volume sensible du détecteur. Ce problème a été discuté par **Herr** *et al.*[23].

La détection d'isotopes émetteurs d'un rayonnement β peut se faire à l'aide de scintillateurs plastiques. **Stranks**[25] a mesuré du gaz carbonique dans une cellule dout l'une des parois est constituée par un scintillateur plastique. **Grandy et Koch**[26] ont réalisé une chambre à circulation de gaz en lucite, placée sur un photomultiplicateur par l'intermédiaire d'un scintillateur plastique "Pilot B". **Funt et Hetherington**[27] ont décrit l'emploi d'un tube capillaire en plastique scintillant.

4. *Utilisation de chambres d'ionisation*

Ce type de détecteur associé à une colonne chromatographique a été utilisé pour la première fois par **Wilzbach et Riesz**[28, 29] et développé principalement par **Cacace et Inam-Ul-Haq**[30, 31]. Le gaz sortant de la colonne traverse une cellule de conductivité thermique et après avoir été dilué par un courant d'azote dans un mélangeur approprié, pénètre dans une chambre d'ionisation en acier inox avec un débit bien déterminé. Ceci a le double avantage d'assurer une bonne stabilité de la chambre d'ionisation dont le fonctionnement dans ce cas est indépendant du débit du gaz vecteur, et d'éviter le chauffage de la chambre, les gaz issus de la colonne étant dilués dans un volume relativement important. La sensibilité de ce dispositif est proportionnelle au rapport entre le volume de la chambre et le débit du gaz, mais le volume de la chambre d'ionisation doit être comparable à celui de la cellule de conductivité thermique. Le courant d'ionisation est mesuré à l'aide d'un électromètre à condensateur vibrant muni d'une résistance d'entrée de 10^{11} Ω. Ce dispositif permet la détection de quelques mμC et la mesure de 20 mμC.

L'analyse de substances à point d'ébullition élevé nécessite le chauffage de la chambre d'ionisation, ce qui peut entraîner l'apparition d'un bruit de fond incompatible avec la mesure de faibles activités et une variation des caractéristiques de la chambre.

MASON *et al.*[32] ont cependant construit une chambre d'ionisation dont le fonctionnement est satisfaisant jusqu'à 240°, l'isolant principal étant en téflon. DOBBS[33] décrit également une chambre fonctionnant à 190° et donnant une bonne réponse avec 0.5 μC de tritium.

De leur côté, CACACE *et al.*[31] ont tourné la difficulté en brûlant les gaz à la sortie de la colonne dans un tube de quartz rempli d'oxyde de cuivre. L'eau formée dans la combustion est adsorbée; le gaz carbonique et l'azote, gaz vecteur, traversent le catharomètre dont la température est maintenue à 0° pour avoir une sensibilité maximale, et après dilution par un courant d'azote, pénètrent dans la chambre d'ionisation. Cette dilution a pour avantage d'augmenter la sensibilité de la chambre puisque le débit y est plus rapide et de garder, ici encore, un débit constant dans la chambre quel que soit le débit du gaz vecteur dans la colonne. La sensibilité du détecteur est 0.5 mμC, et la limite de sensibilité de mesure 2 mμC.

Les différents dispositifs de détection sont résumés dans le Tableau I.

TABLEAU I

LES DIFFÉRENTS DISPOSITIFS DE DÉTECTION

Détecteur	*Efficacité de détection du ^{14}C et du T*	*Observations*	*Références*
Compteur de Geiger	^{14}C: faible T: nulle	Echantillon extérieur au compteur. Température d'emploi limitée.	14–17
Compteur proportionnel à circulation	env. 100%	Les gaz traversent le compteur. Fonctionnement correct jusqu'à 200°. Possibilité d'interférence de "poisons". Très utilisé.	13, 18–20
Scintillateur NaI (Tl)	nulle	Utilisé en analyse continue d'émetteurs γ	2, 8, 22–24
Scintillateur plastique	^{14}C: 60% T: 10%	Peut être attaqué par certaines vapeurs organiques. Températures d'emploi limitées.	25–27
Scintillateur organique et liquide	^{14}C: 75% T: 20%	Utilisé comme détecteur intégral.	9–12
Chambre d'ionisation	env. 100%	Très utilisée. Fontionnement à température élevée dépend de la qualité de l'isolant.	28–33

3. APPLICATIONS DE LA RADIOCHROMATOGRAPHIE EN PHASE GAZEUSE

Les applications de la radiochromatographie en phase gazeuse sont nombreuses et variées. Un grand nombre d'auteurs ont utilisé la technique radiochromatographique dans différents domaines groupés dans le Tableau II. Les références indiquées sont loin d'être complètes et ne sont données qu'à titre d'exemple parmi les travaux les plus récents.

TABLEAU II

APPLICATIONS DE LA RADIOCHROMATOGRAPHIE EN PHASE GAZEUSE

Domaines d'applications	*Références*
Séparation des isotopes de l'hydrogène: H_2, HT, T_2	37, 38
Séparation de gaz rares radioactifs	35, 36
Étude des halogènes de recul (^{80}Br, ^{82}Br, ^{38}Cl, ^{128}I) produits par réactions (n, γ)	1, 2, 6, 8, 23
Étude du tritium formé par les réactions nucléaires ^{6}Li(n,α)T et ^{3}He(n,p)T	39–43
Étude des atomes de ^{14}C formés par la réaction nucléaire ^{14}N$(n,p)^{14}$C	44, 45
Réactions d'échange autoinduit avec le tritium (méthode de marquage de WILZBACH)	10, 28, 29, 46–50
Analyse de produits marqués par des ions ^{14}C accélérés	51, 52
Séparation et purification de molécules marquées	53, 54
Radiolyse de composés marqués	55

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J. Chromatog., 6 (1961) 373–380

SEPARATION OF AROMATIC HYDROCARBONS BY GAS CHROMATOGRAPHY

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(Received January 24th, 1961)

The separation and analysis of mixtures of isomeric aromatic hydrocarbons have been of interest for some time. In the past few years, gas chromatography has been found to be a valuable tool for aromatic separations, and certain stationary phases, such as tetrahalophthalate esters[1], and 7:8-benzoquinoline[2], have been found to be particularly useful.

Since it is clear that certain isomers, particularly *m*- and *p*-xylenes, cannot be readily separated on the basis of vapor pressure alone, those stationary phases which effect good separations must exhibit preferential non-ideality of solution with at least one of the isomers. It has already been pointed out that the tetrahalophthalate esters preferentially form a charge-transfer complex with the *p*-xylene[1]. The complex formation accounts for the fact that *p*-xylene emerges after *m*-xylene from a tetrahalophthalate column, despite the fact that *p*-xylene is more volatile than *m*-xylene.

However, the mechanism by which 7:8-benzoquinoline achieves separation apparently has not been satisfactorily explained. It was the purpose of this research to investigate stationary phases which achieve good separation of xylenes, and to determine the reason for their ability to achieve separation.

RESULTS

In the first phase of this investigation, several low-melting compounds which form stable complexes with aromatic hydrocarbons were used as stationary phases. The results are given in Table I.

All stationary phases were 15% by weight on 35–80 mesh firebrick. The column used was 1 m long by 0.2 in. diameter. Helium was used as the carrier gas.

It is seen that the dinitrotrichlorobenzene gives a separation similar to those achieved by the tetrahalophthalates. However, the separation is not very good. Since this type of separation depends on a selective complexing of the substrate with *p*-xylene, the more volatile component, and since there seems to be no ready means of predicting this selectivity from structural considerations, this type of substrate was not investigated further.

Since both phenanthrene and 7:8-benzoquinoline had been found to give good

TABLE I

COMPLEXING-TYPE STATIONARY PHASES

Compound	Column temp. °C	Ratio of retention volumes p-xylene/m-xylene
3,5-Dinitro-1,2,4-trichlorobenzene	78*	1.04
	99**	1.03
5-Nitro-1,2,4-trichlorobenzene	78	slight separation
Picryl chloride	92	no separation
3,5-Dinitrobenzonitrile	131	no separation
2,4-Dinitro-1,3,5-trichlorobenzene	about 130	no separation

* The compound contained a small amount of mononitrotrichlorobenzene, and melted at about 70°.

** Pure compound.

TABLE II

NON-COMPLEXING-TYPE STATIONARY PHASES

Compound	Column temp. °C	Ratio of retention volumes m-xylene/p-xylene
1,8-Diaminonaphthalene	82	1.093
m-Phenylenediamine	72	1.089
2,6-Dimethoxyphenol	72	no separation
1-Aminoanthracene	115	no separation
2,3,5,6-Tetramethylphenol	96	1.05
N,N-Dimethyl-*p*-phenylenediamine	70	no separation
2,4-Tolylenediamine	104	no separation
2,6-Diaminopyridine	125	1.05
Dihydrophenanthrene	59	about 1.05
Diphenylene oxide	97	no separation
Fluoranthene	114	no separation
Dihydropyrene	140	no separation
1,2-Diaminonaphthalene	99	no separation
1-Naphthylisothiocyanate	70	no separation
m-Bis(*m*-phenoxyphenoxy)benzene	64	1.05
1,4-Dibromonaphthalene	74	no separation
9-Methylanthracene	88	no separation
N,N-Dimethyl-1-naphthylamine	48	about 1.03
4,4′-Dibromodiphenyl ether	68	no separation
1-Ethoxynaphthalene	48	about 1.06
Bardol "B"*	48	1.06
BRV Rubber Softener*	48	about 1.06
Bardol Rubber Compounding Oil*	48	about 1.06
3:4-Benzacridine	110	no separation
Aromatic concentrate**	48	no separation
Acridine	109	no separation

* Product of Barrett Division, Allied Chemical Corporation.

** Product of Enjay Chemical Company.

separations of the xylenes, a number of other aromatic compounds, usually with electron-donating substituents, were investigated as stationary phases. The results are given in Table II.

These stationary phases were tested under the same conditions as those reported in Table I. Since the Bardol Rubber Compounding Oil gave a good separation, it was fractionally distilled into about 20 fractions. Nine of these fractions were tested as stationary phases. None gave a better separation than the original mixture.

Fig. 1 shows the separation of *m*- and *p*-xylenes achieved by use of a 0.2 in. × 3.5 m column packed with 15% 1,8-diaminonaphthalene on 35–80 mesh firebrick, at 68°. The flow rate of helium was 82 ml/min. This stationary phase was also tested for separation of ethylbenzene and *p*-xylene. It was found that the ratio of retention volumes of *p*-xylene and ethylbenzene was 0.991, at 68°.

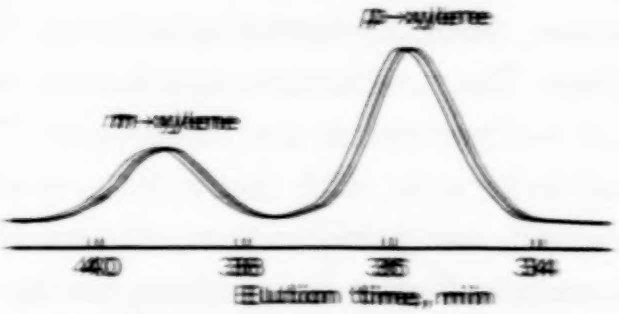

Fig. 1. Separation of xylene isomers.

DISCUSSION

Since phenanthrene and 7:8-benzoquinoline had been found to serve as satisfactory substrates for the separation of the xylenes, it seemed possible that their aromatic character was responsible for their behavior. At least two possible aspects of aromaticity could be the real cause, conjugation or polarizability. Accordingly, the substrates tested in this study were chosen from among aromatic compounds with high conjugation, and those with high polarizability (high refractive index). It is clear from the results that conjugation as such is not important. All of the non-complexing compounds serving as satisfactory substrates do have a high refractive index. However, no immediate correlation between resolving power and refractive index is apparent.

All of the non-complexing compounds serving as satisfactory stationary phases show preferential retention of *m*-xylene, since the ratio of the vapor pressure of *p*-xylene to that of *m*-xylene is about 1.02–1.03 at the temperature of interest. Since *m*-xylene is not being retained by complex formation the separation is due to preferential repulsion between *p*-xylene and the stationary phase. It seems most reasonable to attribute this repulsion to an interaction between the π-electrons of the stationary phase and those of the *p*-xylene. The presence of electron-donating groups and a high degree of resonance in the stationary phase would tend to increase the π-electron repulsion, and the refractive index. However, steric factors would also seem to be important, because of the lack of correlation of activity with refractive index.

This interpretation is strengthened by the fact that *p*-xylene is eluted from 1,8-diaminonaphthalene before ethylbenzene. This most unusual result is also due to the selective repulsion between the stationary phase and the *p*-xylene. This repulsion leads to a change of the activity coefficient of *p*-xylene, relative to that of the ethylbenzene, and this change is about 8%. The change with respect to *m*-xylene is about 6% in the same direction, and it seems reasonable that *m*-xylene is repelled by the stationary phase, relative to ethylbenzene.

It has recently been reported[3] that bis(phenoxyphenyl) ether shows a preferential repulsion for *p*-ethyltoluene with respect to *m*-ethyltoluene. This fact is consistent with the foregoing discussion, since the homologous ether has shown a slight preferential repulsion for *p*-xylene. The 1,8-diaminonaphthalene was tested as a stationary phase for the separation of *o*-ethyltoluene and mesitylene. The ratio of the retention volumes at 68° was found to be 1.11, with mesitylene emerging first. A moderately good separation was achieved, using only a 1-m column. It would appear that the stationary phase exhibits a marked selective repulsion for the mesitylene, since this is a very close-boiling pair. It would be expected that the 1,8-diaminonaphthalene would give a much better separation for the *m*- and *p*-ethyltoluenes than does the bis(phenoxyphenyl) ether, in view of the above discussion.

SUMMARY

Various compounds were tested as stationary phases for the separation of xylene isomers by gas chromatography. Both 1,8-diaminonaphthalene and *m*-phenylenediamine were found to give good separations. A number of other compounds were found which gave some separation.

All of the compounds known which give good separations of the *m*- and *p*-xylenes, such as phenanthrene, 7:8-benzoquinoline, 1,8-diaminonaphthalene, and *m*-phenylenediamine, appear to function by preferential repulsion of the *p*-xylene. This repulsion is attributed to an interaction between the π-electrons of the stationary phase and those of the *p*-xylene.

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THE SELECTIVE SEPARATION OF HIGH-BOILING AROMATIC COMPOUNDS

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(Received March 1st, 1961)

In connection with a research project involving the pyrolysis of simple model compounds containing some of the groupings thought to occur in coal, separations were required of products boiling at temperatures up to 350°. It proved impossible to separate several of the components with Apiezon L as the stationary phase, and a change was therefore made to a more selective alternative, namely Reoplex 400, which has recently been used by Janák and Hřivnáč[1]. In the course of the work several interesting selectivity relationships were observed due to "π"-electron interactions of the type discussed by the above authors and by Langer, Zahn and Pantazoplos[2], who have recently separated *m*- and *p*-xylene using di-*n*-propyl tetrachlorophthalate as the stationary phase.

EXPERIMENTAL

A modified Pye gas–liquid chromatograph incorporating an argon ionization detector was used for determining the relative retention volumes. The inlet system was modified by introducing a sampling valve based on the design described by Tenney and Harris[3]. The inlet gas pressure was controlled by means of a Sunvic Nullmatic regulator, and temperature cycling of the column heater was minimized by connecting a variable resistor across the temperature regulator to provide a suitable base load for the heater. The argon flow rate throughout the study was 100 ml/min.

The copper chromatographic columns were 4 ft. long and were packed with 5% Apiezon L on Embacel and with 5% Reoplex 400 on Embacel. To ensure accurate comparisons, the same amount of packing was provided on each column.

In one case, retention data for the *ω,ω'*-diphenylalkanes were determined on a 6 ft. column of 25% Apiezon L using a Griffin and George Mark II chromatograph with hydrogen as the flow gas (4.0 l/h).

All the quoted relative retention values were means of five injections for each substance on each stationary phase.

DISCUSSION

Table I shows the relative retention volumes of a series of ω,ω'-diphenylalkanes ($C_6H_5[CH_2]_nC_6H_5$) on Apiezon L (5%) and Reoplex 400 (5%). For comparison, similar retention data for Apiezon L (25%) are included which, although not strictly comparable, indicate that the relationships were unaffected by the amount of stationary phase present.

TABLE I

RETENTION DATA OF ω,ω'-DIPHENYLALKANES

Diphenylalkane	*n*	*B.p. °C*	*Relative retention volumes*		
			Apiezon L (5%) 180°	*Reoplex 400 (5%) 180°*	*Apiezon L (25%) 200°*
Diphenyl	0	255	0.881	0.941	0.868
Diphenylmethane	1	265	1.00	1.00	1.00
Dibenzyl	2	284	1.51	1.32	1.47
1,3-Diphenylpropane	3	302	2.55	2.00	2.48
1,4-Diphenylbutane	4	317	3.95	2.77	3.76
1,6-Diphenylhexane	6	348	10.38	5.22	9.06

Plots of the logarithms of these retention data against boiling point for each stationary phase are shown in Figs. 1–3. The curves are essentially linear except for the lower members of the series, namely diphenyl and diphenylmethane. Their retention times indicate them to be more strongly retained, relative to their boiling-points, than the other diphenylalkanes. Such behaviour is evidence for polarizability, and the effect is especially pronounced on Reoplex 400, a polar stationary phase (compare the slopes of the relevant portions of each curve).

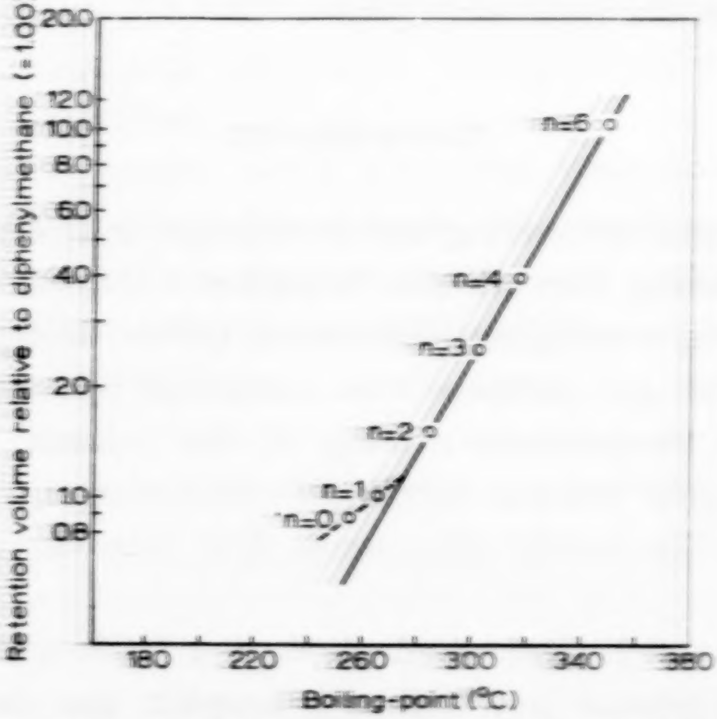


Fig. 1. Log retention volume *vs.* boiling-point. Sample: ω,ω'-diphenylalkanes ($C_6H_5[CH_2]_nC_6H_5$); column: 4ft., 5% Apiezon L on Embacel; temperature: 180°; Pye apparatus; flow rate: 100 ml/min; pressure (inlet): 26.5 lb./sq.in.

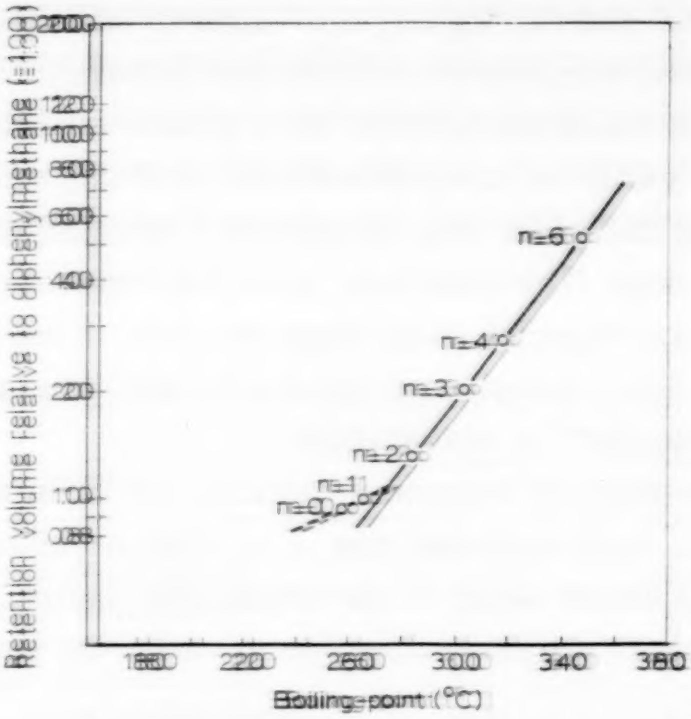


Fig. 2. Log retention volume *vs.* boiling-point. Sample: ω,ω′-diphenylalkanes ($C_6H_5[CH_2]_nC_6H_5$); column: 4 ft., 5% Reoplex 400 on Embacel; temperature: 180°; Pye apparatus; flow rate: 100 ml/min; pressure (inlet) 29.5 lb./sq.in.

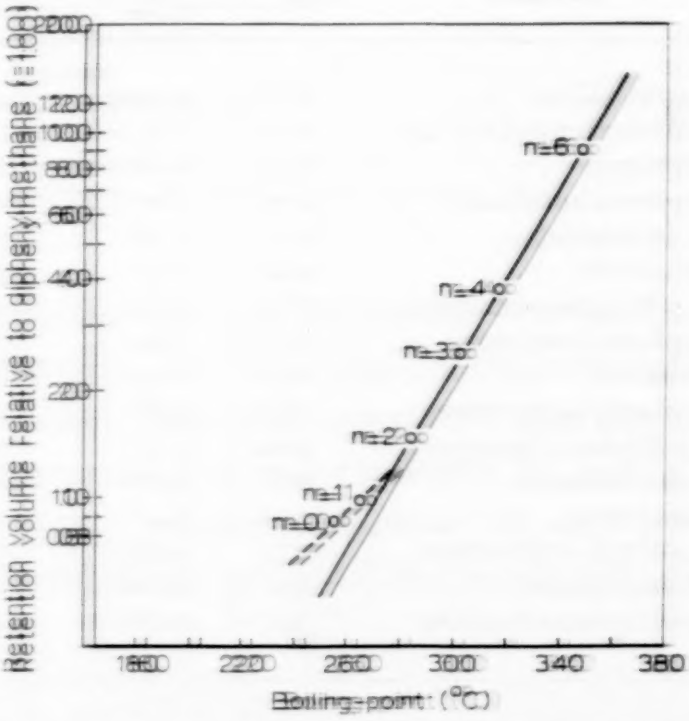
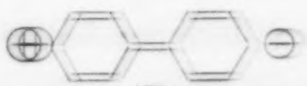


Fig. 3. Log retention volume *vs.* boiling-point. Sample: ω,ω′-diphenylalkanes ($C_6H_5[CH_2]_nC_6H_5$); column: 6 ft., 25% Apiezon L on Embacel; temperature: 200°; Griffin and George Mark II apparatus; flow rate: 4.0 l/h.

This revealed polarizability is not surprising because, in both diphenyl and diphenylmethane, the phenyl groups interact electronically, as their ultra-violet spectra[4] show. With diphenyl, the ultra-violet spectrum is unlike that of benzene and is due to the contribution of such dipolar structures as (I).

Similarly, the ultra-violet spectrum of diphenylmethane[4], while not very dissimi-



(I)

lar from that of dibenzyl and the higher, ω,ω'-diphenylalkanes, does show differences which may be attributed to electronic interaction between the benzene rings across the central carbon atom. CRAM AND ANTAR[5] have shown that diphenylmethane can be acylated to give high yields of monosubstituted product, indicating that the substituent is deactivating the other ring, presumably because of electronic interaction between the benzene rings. This contrasts with the behaviour of dibenzyl and the higher ω,ω'-diphenylalkanes which, even when one mole of acylating agent is employed, give mixtures of non-, mono- and bis-substituted material because the rings are electronically independent of one another.

Table II lists the relative retention volumes for a number of representative high-boiling aromatic hydrocarbons (the ω,ω'-diphenylalkanes are included, for comparison). Table III shows some of the separation factors which can be derived from Table II. In brackets are the values found earlier by JANÁK AND HŘIVNÁČ[1].

TABLE II

RELATIVE RETENTION VOLUMES AT 180° FOR HIGH-BOILING AROMATIC COMPOUNDS

Compound	*B.p.* °C	*Apiezon L* (5%)	*Reoplex 400* (5%)
Naphthalene	218	0.409	0.436
1-Methylnaphthalene	245	0.732	0.726
Diphenyl	255	0.881	0.941
Diphenylmethane	265	1.00	1.00
Acenaphthene	277	1.58	1.50
Dibenzyl	284	1.51	1.32
3:4-Benzocoumarone	285	2.23	2.90
Diphenylene oxide	287	1.92	1.98
Fluorene	297	2.43	2.66
Diphenylacetylene	297	3.06	2.75
1,3-Diphenylpropane	302	2.55	2.00
trans-Stilbene	307	3.86	4.27
Xanthene	308	3.18	3.10
1,4-Diphenylbutane	317	3.95	2.77
Phenanthrene	338	6.20	7.24
1,6-Diphenylhexane	348	10.38	5.22

Tables II and III reveal the strong "π"-electronic interaction which exists between Reoplex 400 and certain aromatic compounds. Of special interest is the reversal of order shown by the pair phenanthrene/1,6-diphenylhexane on Reoplex 400 compared with their order on Apiezon L. The strong retention of phenanthrene on Reoplex 400 is undoubtedly a consequence of the localization of "π"-electrons at the 9,10-bond, conferring on that bond a marked double-bond character.

Similarly, the pairs fluorene/1,3-diphenylpropane and *trans*-stilbene/1,4-diphenylbutane are inseparable on Apiezon L, but are readily separated on Reoplex 400. The ω,ω'-diphenylalkanes, where $n \geqslant 2$, suffer no enhanced sorption on Reoplex 400 because the aromatic rings act independently and "π"-electron localization is minimal. Both fluorene and *trans*-stilbene have structures in which "π"-electron localization occurs, the former at the methylene bridge and the latter at the central double bond joining the phenyl nuclei. Similarly, the pair acenaphthene/dibenzyl, both of which have ethane bridges, are separated slightly more easily on Reoplex 400. This is not surprising, because the naphthalene nucleus of acenaphthene will confer more polarizability on its molecule than do the two isolated benzene nuclei on dibenzyl.

TABLE III

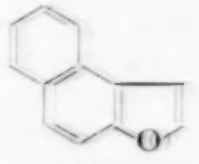
SEPARATION FACTORS (DERIVED FROM TABLE II)*

Compound	*Apiezon L (5%) 180°*	*Reoplex 400 (5%) 180°*
Diphenylmethane/diphenyl	1.14	1.06
Dibenzyl/diphenylmethane	1.51	1.32
Fluorene/acenaphthene	1.54 (1.51)	1.77 (1.77)
Fluorene/diphenylene oxide	1.27 (1.38)	1.33 (1.23)
Fluorene/1,3-diphenylpropane	0.95	1.33
1-Methylnaphthalene/naphthalene	1.79 (1.78)	1.66 (1.62)
trans-Stilbene/1,4-diphenylbutane	0.98	1.54
Diphenylene oxide/acenaphthene	1.21 (1.09)	1.32 (1.44)
3:4-Benzocoumarone/diphenylene oxide	1.16	1.46
Acenaphthene/dibenzyl	1.05	1.14
Phenanthrene/1,6-diphenylhexane	0.60	1.39
Diphenylacetylene/fluorene	1.26	1.03
Xanthene/fluorene	1.31	1.17
Xanthene/1,4-diphenylbutane	0.80	1.12

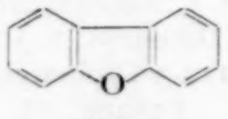
* Values in parentheses are those of JANÁK AND HŘIVNÁČ[1].

Our separation factors for the pair diphenylene oxide/acenaphthene differ from those of JANÁK AND HŘIVNÁČ[1]. We were able to separate these substances readily on Apiezon L, and we believe that this separation was due to the use of a more homogeneous support (Embacel) than was employed by JANÁK AND HŘIVNÁČ.

An interesting pair are 3:4-benzocoumarone (II) and diphenylene oxide (III). They are separated fairly readily on Apiezon L but much more easily on Reoplex 400.



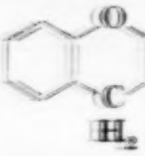
(II)
Boiling-point 285°



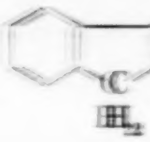
(III)
Boiling-point 287°

The "outside" furan ring of 3:4-benzocoumarone contains a styrene-type double bond where "π"-electrons are localized, and increased sorption on Reoplex 400 results. The polarization accorded to the diphenylene oxide molecule by the oxygen atom is obviously not as strong as that induced by the "π"-electron localization at the 1,2-bond of 3:4-benzocoumarone.

The polarizability of xanthene (IV) is shown by its comparison with 1,4-diphenylbutane, there being a reversal of order on Reoplex 400 compared with the order on Apiezon L. This polarizability is somewhat less than that of fluorene (V), as a comparison of their separation factors (Table III) reveals. The oxygen bridge in xanthene interrupts conjugation between the aromatic nuclei and lowers the polarizability. Table II reveals that, with Apiezon L, 3:4-benzocoumarone, diphenylacetylene and *trans*-stilbene behave anomalously in that they are not eluted linearly with respect to their boiling-points. These anomalies may be due to the high degree of unsaturation present in these compounds. Diphenylacetylene also shows anomalous behaviour on Reoplex 400, for its separation factor with respect to fluorene is not in keeping with the high degree of polarization suggested by its sorption on Apiezon L. This anomalous behaviour on Apiezon L is also readily seen in Fig. 4, in which the logarithms of the relative retention data for the compounds in Table III (5% Apiezon L at 180°) are plotted against boiling-point. A straight line can be drawn through the points for most of the compounds except 3:4-benzocoumarone, diphenylacetylene and *trans*-stilbene, which are on a different straight line, and the ω,ω'-diphenylalkanes (dotted line).



(IV)



(V)

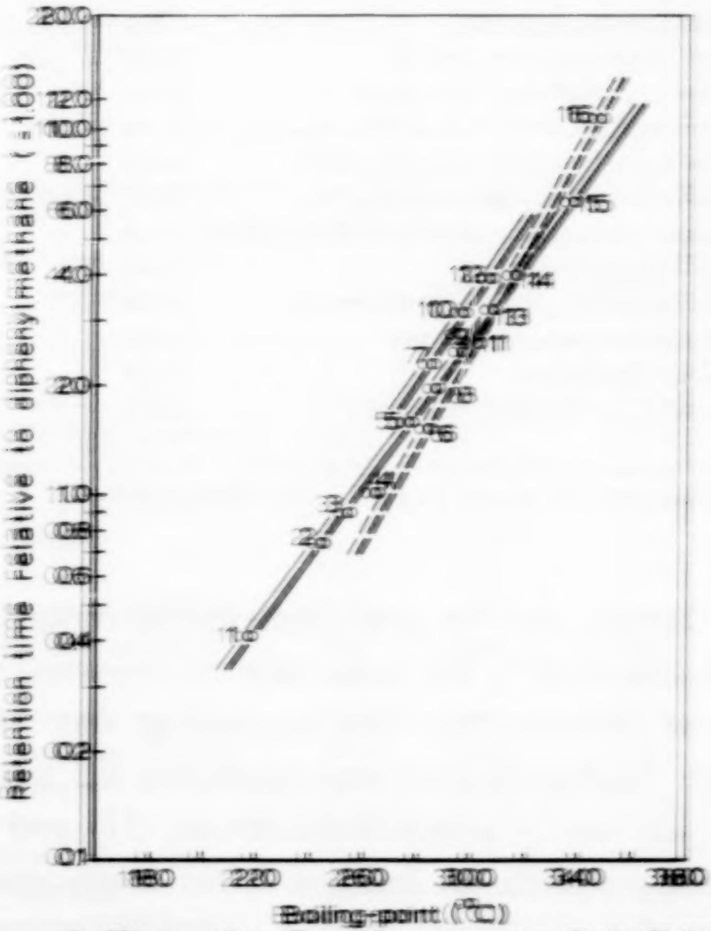


Fig. 4. Log retention volume *vs.* boiling-point. Sample: compounds in Table III; column: 4 ft., 5% Apiezon L on Embacel; temperature: 180°; Pye apparatus; flow rate: 100 ml/min; pressure (inlet): 26.5 lb./sq.in. 1. Naphthalene; 2. 1-Methylnaphthalene; 3. Diphenyl; 4. Diphenylmethane; 5. Acenaphthene; 6. Dibenzyl; 7. 3:4-Benzocoumarone; 8. Diphenylene oxide; 9. Fluorene; 10. Diphenylacetylene; 11. 1,3-Diphenylpropane; 12. *trans*-Stilbene; 13. Xanthene; 14. 1,4-Diphenylbutane; 15. Phenanthrene; 16. 1,6-Diphenylhexane.

Comparing the sorption behaviour of those hydrocarbons which are eluted linearly with respect to their boiling-points on 5% Apiezon L (Fig. 4) with their corresponding behaviour on 5% Reoplex 400, and assuming that polarizability is the sole cause of such selective sorption effects, the following empirical series of decreasing polarizability can be suggested: phenanthrene > fluorene > naphthalene = diphenyl > 1-methylnaphthalene > diphenylene oxide > xanthene > acenaphthene.

However, on the basis of molar refraction measurements, Schuyer, Blom and Van Krevelen[6] obtained satisfactory agreement between theoretical calculations and experimental results for the following series of decreasing polarizability:

phenanthrene > fluorene > acenaphthene > naphthalene,

the members acenaphthene and naphthalene being transposed. The position of 1-methylnaphthalene in our series is also noteworthy, as Syrkin and Dyatkina[7] indicate 1-methylnaphthalene to be more polarizable than naphthalene while pointing out, however, that molar refraction data are difficult to interpret. It would appear that while selective sorption behaviour is probably mainly a polarizability phenomenon, there may be other factors involved.

Similar differences in selective sorption were observed with several low-boiling aromatic compounds. Their relative retention volumes are listed in Table IV, and some of the derived separation factors are compared in Table V. Since 5% Apiezon L retains a given sample longer than 5% Reoplex 400 for the same length of column, the two phases were investigated at different temperatures, namely, 110° for Apiezon L and 80° for Reoplex 400. Therefore, the separation factors quoted in Table V are for those pairs of compounds where the selective sorption effect is significantly large.

TABLE IV

RELATIVE RETENTION VOLUMES FOR VARIOUS AROMATIC COMPOUNDS

Compound	*B.p.* °*C*	*Relative retention volumes*	
		5% Apiezon L (110°)	*5% Reoplex 400 (80°)*
Cumene	152.4	0.460	0.195
n-Propylbenzene	159.5	0.553	0.223
α-Methylstyrene	165.5	0.780	0.426
Coumarone*	171.4	1.00	1.00
Hydrindene	177	1.15	0.509
Indene*	182.8	1.40	0.928
n-Butylbenzene	183	1.09	0.400
Tetralin	207.4	2.66	1.16
Naphthalene	218	3.97	3.22
Thionaphthene*	222	4.91	4.29

* These compounds are in Series 1 (see page 393).

The most interesting comparison here is that of hydrindene (hydro-aromatic) and *n*-butylbenzene (open-chain). These hydrocarbons emerge together on Apiezon L, but are readily separated on Reoplex 400. On the basis of the previous arguments, this selectivity must be due to the fact that hydrindene is more polarizable than *n*-butylbenzene.

TABLE V

COMPARISON OF SEPARATION FACTORS (DERIVED FROM TABLE IV)

Compounds	*Separation factor*	
	5% Apiezon L (110°)	*5% Reoplex 400 (86°)*
Indene/coumarone	1.40	0.98
Hydrindene/indene	1.22	1.80
Hydrindene/*n*-butylbenzene	1.06	1.27
Coumarone/hydrindene	0.87	2.00
Coumarone/*n*-butylbenzene	0.91	2.50
Tetralin/naphthalene	1.49	2.78
Coumarone/thionaphthene	4.91	4.29

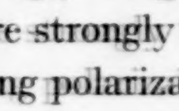
This increased polarizability is paralleled by the increased reactivity toward electrophilic substitution of hydrindene in the aluminium chloride-catalysed benzoylation reaction[8]. Although rate figures are not quoted for *n*-butylbenzene, the rate for hydrindene is approximately 28 times that of ethylbe[illegible]ne[8,9] and presumably greater than that of *n*-butylbenzene to the same degree.

The marked difference in the separation factors of the pair hydrindene/indene is clearly due to the unsaturated linkage in the five-membered ring of indene. This linkage, being a "styrene-type" double bond, interacts strongly with Reoplex 400. Whereas coumarone and indene are easily separated on Apiezon L, no separation was observed on Reoplex 400, indicating that coumarone is retained more strongly on Reoplex 400. Evidently, therefore, coumarone is more polarizable than indene.

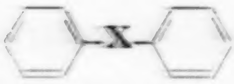
In view of these selective sorption effects, it was not surprising that naphthalene and tetralin showed marked differences of behaviour on Reoplex 400, the former being retained more strongly. Naphthalene is more fully aromatic and its "π"-electron cloud is held more strongly than that of the hydroaromatic tetralin.

The order of decreasing polarizability for the compounds in Table IV is: coumarone > naphthalene > indene > hydrindene = tetralin > *n*-butylbenzene. In general, the order of retention is aromatic > hydroaromatic > open-chain for Reoplex 400.

Since there appears to be a qualitative relationship between the degree of polarizability of aromatic compounds and sorption on a polar phase, the following series were examined on both 5% Apiezon L and 5% Reoplex 400:

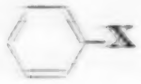
Series 1  where X = CH_2, O, S (Table IV)

Series 2



where X = CH_2, O, S, NH (Table VI)

Series 3



where X = CH_2CH_3
= $CH = CH_2$
= $C{\equiv}CH$
= $CH_2{\cdot}CH_2{\cdot}CH_3$ (Table VII)

Series 1. Examination of the relative retention volumes for indene (X = CH_2), coumarone (X = O), and thionaphthene (X = S) illustrates the effect of the hetero-atom in conferring polarizability on the common nucleus. Oxygen in this series has a noticeably greater influence than sulphur, indicating coumarone to be more polarizable than thionaphthene, in agreement with the known reactivities of both compounds, coumarone being the more reactive[10]. Consistent figures for indole (X = NH) could not be obtained because of its marked asymmetrical peak on both 5% phases, presumably because of the acidity of the –NH– group.

Series 2. The degree of decreasing polarizability for this series is diphenylamine > diphenyl ether > diphenyl sulphide = diphenylmethane. Diphenylamine is recorded as an asymmetrical peak on both phases, but is much more strongly retained on Reoplex 400—a measure of the polarizability of the molecule.

Diphenyl ether is somewhat more polarizable than either diphenyl sulphide or diphenylmethane, which in this respect are practically equivalent. The oxygen atom confers a slightly greater degree of polarizability on the molecule than does the sulphur atom. However, selective sorption effects are not so pronounced as in Series 1, there being only the conjugation of the two benzene nuclei involved across the central atom.

Series 3. It was expected that an olefinic bond would confer enhanced polarizability on a given molecule (compare ethylbenzene and styrene), but the powerful effect of an acetylenic bond (phenylacetylene) was surprising. Phenylacetylene gave a strong asymmetrical peak, presumably because of its acidic hydrogen; and, in addition, its sorption on the non-polar Apiezon L is not in accord with its boiling-point (such behaviour is reminiscent of diphenylacetylene (Table II)). The magnitude of its relative retention volume on 5% Reoplex 400 is considerably greater than that of styrene, indicating higher polarizability.

TABLE VI

RELATIVE RETENTION VOLUMES AT 140° FOR COMPOUNDS OF SERIES 2

Compound	*B.p.* °C	5% *Apiezon L*	5% *Reoplex 400*
Diphenyl ether (X = O)	259	0.801	0.925
Diphenylmethane (X = CH_2)	265	1.00	1.00
Diphenyl sulphide (X = S)	296	2.35	2.49
Diphenylamine (X = NH)	302	3.10	12.46

There is an interesting selectivity effect in the behaviour of ethylbenzene and *n*-propylbenzene on both phases. The separation factors for these compounds are 2.14 and 1.82 on Apiezon L and Reoplex 400 respectively. Ethylbenzene is therefore more polarizable than *n*-propylbenzene, a result which is in accord with the respective electronegativities of these alkyl substituents.

Often in the pyrolyses of hydroaromatics or alkylaromatics, *o*-xylene is a product. On Apiezon L there is not much separation of *o*-xylene from styrene, a frequent accompanying pyrolysis product, especially if the amount of *o*-xylene is small. From Table VIII it can be seen that Reoplex 400 is a selective phase for the easy separation of these two substances.

TABLE VIII

RELATIVE RETENTION VOLUMES AT 50° OF VARIOUS LOW-BOILING AROMATIC COMPOUNDS

Compound	*B.p.* °*C*	5% *Apiezon L*	5% *Reoplex 400*
Ethylbenzene	136	1.00	1.00
Phenylacetylene	142	1.95	3.67
o-Xylene	144.4	1.48	1.53
Styrene	146	1.70	2.30
n-Propylbenzene	159.5	2.14	1.82

Finally, it should be emphasized that, when the substances under investigation were either fairly acidic or basic, asymmetrical peaks, each with a long trailing edge, were recorded on these columns with 5% stationary phase. Such departure from ideal conditions was shown by phenol, thiophenol, aniline, diphenylamine, indole, quinoline and carbazole. Since the departure from ideal conditions was less on 25% stationary phase, reaction with the support may be responsible.

ACKNOWLEDGEMENTS

We are indebted to Mr. H. R. BROWN, Chief, Division of Coal Research, C.S.I.R.O., for his interest and support, and to Geigy (Australasia) Pty. Ltd., Botany, New South Wales, for the Reoplex 400 used in the investigation.

SUMMARY

The selective sorption of various aromatic compounds boiling at temperatures up to 350° was studied on a polar phase (Reoplex 400). Comparison of their behaviour on the non-polar Apiezon L indicates that this selectivity may be explained by "π"-electron interaction with the stationary phase (acceptor). While a qualitative estimate of the degree of polarizability of the compounds under investigation could thereby be obtained, sufficient cases of anomalous sorption exist to indicate that factors other than polarizability may be responsible for selective sorption.

From the practical standpoint, Reoplex 400 is a suitable selective stationary phase for the separation of many aromatic hydrocarbons which normally emerge together on non-polar phases. Reoplex 400 retards such hydrocarbons in the order aromatic > hydroaromatic > open-chain.

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GRUPPENMÄSSIGE IDENTIFIZIERUNG NICHTFLÜCHTIGER AROMATISCHER STOFFE MIT HILFE DER GASCHROMATOGRAPHIE

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(Eingegangen den 30. Januar 1961)

Zur Identifizierung von organischen Stoffen werden die verschiedensten analytischen Methoden angewandt, nicht in letzter Linie die Papier- und Gas-Chromatographie. Die Gaschromatographie bleibt aber nur auf solche Stoffe beschränkt, die man bei höheren Temperaturen in Dämpfe überführen kann. In letzter Zeit zeigte sich aber auch die Möglichkeit selbst nichtflüchtige Stoffe mittels Gaschromatographie zu identifizieren. Die Identifizierung beruht auf der Erscheinung, dass sich diese Stoffe bei höheren Temperaturen pyrolytisch spalten lassen. Die gasförmigen Produkte lassen sich gaschromatographisch trennen und mit dem Chromatogramm eines in gleicher Weise gespaltenen Standardstoffes vergleichen.

In der analytischen Chemie sind schon früher einige Arbeiten erschienen, die sich mit der Identifizierung organischer Stoffe mittels Spaltung beschäftigten. So führt z.B. FEIGL[1] den mikroanalytischen Nachweis von Wasserstoff in organischen Stoffen mittels Pyrolyse in Gegenwart von Schwefel durch. Er bestimmt den entstandenen Schwefelwasserstoff. Derselbe Autor[2] verwendet die Pyrolyse und wasserentziehende Stoffe zum Nachweis von Stickstoff und Sauerstoff enthaltenden Gruppen oder zum Nachweis von aromatischen und aliphatischen Cyaniden[3]. Durch Kombination der Pyrolyse und wasserentziehenden Stoffen identifiziert dieser Autor[4] auch eine Reihe von organischen Verbindungen. In einer anderen Arbeit beschäftigt sich HARMS[5] mit der Identifizierung komplizierter organischer Materialien unter Benützung der pyrolytischen Produkte, die er spektroskopisch im Infrarot analysiert.

Aber die Verbindung von Pyrolyse mit der Gaschromatographie gestattet eine vielseitigere Anwendung. JANÁK[6] befasst sich mit der pyrolytischen Spaltung einiger Barbiturate und schlägt die Durchführung der Pyrolyse an einem glühenden Platindraht vor. Die Gaschromatographie verwendete auch MARTIN[7] zur Auffindung zerfallender Produkte organischer Stoffe. Dieser benützt aber zur Spaltung U.V.-Strahlung. Die Pyrolyse in Verbindung mit der Gaschromatographie zum Nachweis von Akrylaten und Methakrylaten benützt RADELL[8]. Die Spaltung erfolgt in einem besonderen Kupferrohr das in Wood'schem Metall auf 500° erhitzt wird.

Bei Verfolgung des Verlaufes der pyrolytischen Spaltung verschiedener Stoffe (deren in der Literatur eine grosse Anzahl angeführt wird) kommt man zu folgendem

Schluss: Den Verlauf der Pyrolyse kann man durch Temperatur, durch die Gasatmosphäre in der sie verläuft, und durch Zusatz verschiedener Katalysatoren zu den zu pyrolysierenden Stoffen beeinflussen.

Um gute Resultate bei der Verbindung von Pyrolyse und Gaschromatographie zu erreichen, ist es nötig, für beide Vorgänge optimale Bedingungen zu wählen. Für die Pyrolyse aromatischer Verbindungen wird es nötig sein eher bei höheren Temperaturen und inerter Atmosphäre zu arbeiten. Die Gaschromatographie hingegen erfordert eher niedrigere Temperaturen und eine optimale Trennung der Gasbestandteile.

EXPERIMENTELLER TEIL

Das Gerät

Die chromatographische Trennung erfolgte in einem Gerät eigener Konstruktion, mit einem Thermostaten ausgestatteten künstlichen Luftzirkulation und mit einer Wärmeleitfähigkeitzelle mit der Möglichkeit bis zu 250° zu arbeiten. Die chromatographische Kolonne ist so angebracht, dass ihr Beginn durch die obere Wand des Thermostaten durchgesteckt ist, so dass man an dieser Stelle die Zugabe der Probe entweder mittels Injektionsspritze (bei normaler chromatographischer Trennung) oder durch Einlegen eines Platinnetzes mit der Probe durchführen kann, worauf die pyrolytische Spaltung erfolgt. Die Wärmeleitfähigkeitzelle steht in Verbindung mit dem Kompensationsschreiber der einen Bereich von 1 mV besitzt (Honywell-Brown).

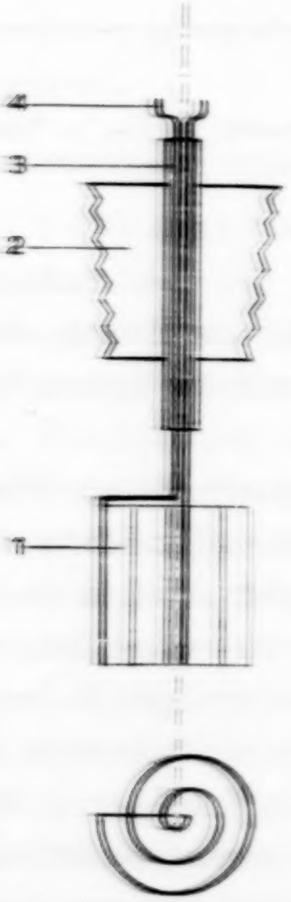


Fig. 1. 1. Platinnetz; 2. Butazinstopsel; 3. Keramische Einlage; 4. Platindrähte.

Zum Unterschied von JANÁK[6] musste beim Probezusatz so vorgegangen werden dass die Wärmeleitfähigkeitzelle ein hinreichend deutliches Signal gab. Das heisst dass die zugesetzte Probe 2–5 mg betragen muss. Auf einen Platindraht eine solche Menge aufzutragen ist nicht möglich und selbst wenn, so wären keine reproduzierbaren Resultate zu erreichen[6].

Deshalb trat an Stelle des Platindrahtes ein Platinnetz aus feinem Gewebe. Das Netz hat die Ausmasse 50 × 25 mm und ist in eine Spirale gewunden (siehe Fig. 1), damit es bequem in die Öffnung der chromatographischen Kolonne eingeführt werden kann. An beiden Enden der Spirale sind 0.5 mm dicke Platindrähte befestigt, welche gleichzeitig die Zuführung des elektrischen Stromes bilden. Diese Drähte durchbrechen eine zweiwegige keramische Einlage welche einen Butazinstopfen trägt. Dieser Stopfen wird beim Versuch mit der Spirale in das offene Ende der chromatographischen Kolonne eingeführt.

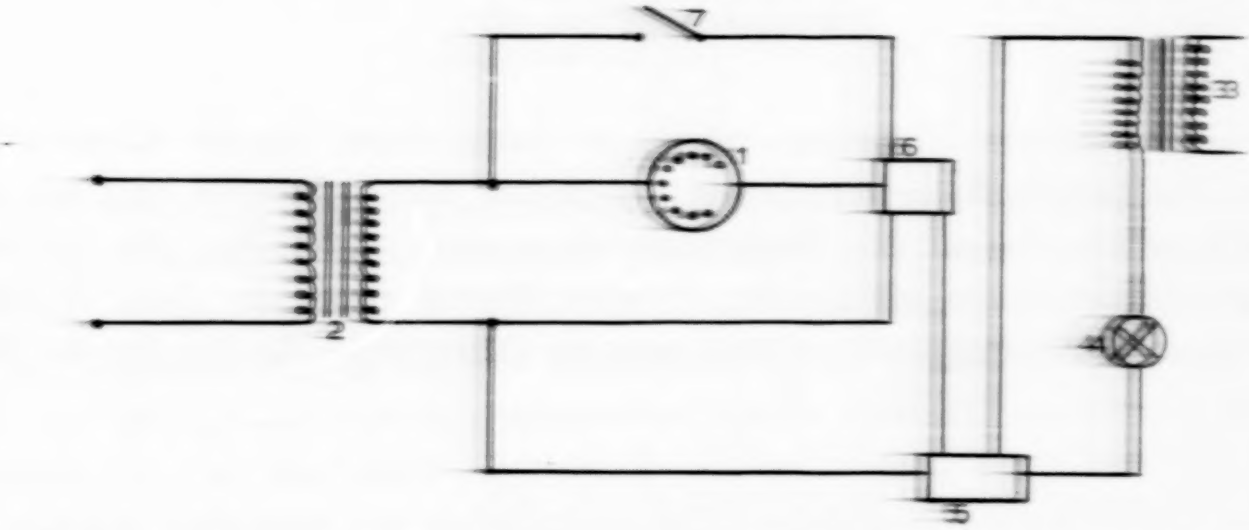


Fig. 2. 1. Telephonwähler; 2. Transformator 220/24; 3. Transformator 220/15–20; 4. Platinnetz; 5. Quecksilberrelais; 6. Telephonrelais; 7. Druckschalter.

Zum Glühen wird das Platinnetz durch Wechselstrom niedriger Spannung gebracht. Als Spannungsquelle dient ein Transformator von 15–20 V. Das Glühen muss aber in genau gleichen Zeitintervallen erfolgen im Bereich von 0–2 Sekunden. Um einen elektronischen Zeitschalter zu vermeiden, haben wir einen gewöhnlichen Telephonwähler verwendet, der es ermöglichte reproduzierbare Zeitintervalle von 0.3–1.4 Sekunden in Abständen von 0.1 Sekunden herzustellen. In Hinsicht darauf, dass der Strom der das Netz durchfliesst genug stark ist (5 A), ist es nötig den Telephonwähler in Verbindung mit einem Quecksilberrelais anzuwenden. Damit es bei einer zufälligen Störung nicht zur Verbrennung des Platinnetzes kommt, ist in den Glühkreis ein Druckschalter angeschaltet (siehe Fig. 2). Das Netz wird von den Resten der pyrolytischen Spaltung durch Glühen an der Luft gereinigt, allenfalls von Zeit zu Zeit durch Glühen mit $KHSO_4$.

Die Länge der Kolonne für die chromatographische Trennung betrug 90 cm, Durchmesser 5 mm, sie war mit aktiver Kohle (Korngrösse 0.4–0.6 mm) gefüllt, Temperatur 27° und Stickstoff als Trägergas von einer Geschwindigkeit von 5.0 l/Stunde.

Pyrolyse organischer Stoffe

Die Durchführung der pyrolytischen Spaltung erfolgte folgendermassen: Der feste, zur Identifizierung bestimmte organische Stoff wird in einem geeigneten flüchtigen Lösungsmittel gelöst (ca. 5%). Die Lösung erfolgt in einem niedrigen breiten Reagensglas in das man das Platinnetz leicht einführen kann. Nach Anschluss des Netzes wird das Lösungsmittel verflüchtigt. Dann wird das Netz in die chromatographische Kolonne eingeführt und auf angegebene Weise zur Glut gebracht. Der Schreiber zeichnet dann das chromatographische Spektrum der Spaltprodukte. Zum Studium der Identifizierung fester organischer Stoffe mittels pyrolytischer Spaltung wählten wir eine Reihe aromatischer Stoffe und zwar verschieden substituierte aromatische Säuren. Die einzelnen gebildeten Pyrolyseprodukte wurden in Tabellen I–IV zusammengefasst. Die Schätzung der Menge der einzelnen Spaltprodukte geschah folgendermassen: — kleine, — — mittlere, — — — grosse Menge.

Pyrolyse organischer Stoffe unter Zusatz eines Katalysators

Der bisher beschriebene Verlauf der pyrolytischen Spaltung geschah ohne Zusatz eines Katalysators. Informativ versuchten wir den Einfluss eines Zusatzes von $CoCl_2$ auf die Spaltung festzustellen. Es wurde eine 5%ige Lösung von $CoCl_2$ in absolutem Alkohol hergestellt und davon wurden 3–4 Tropfen zu 5 ml Substanzlösung, die zur Identifizierung bestimmt war, zugesetzt.

RESULTATE UND DISKUSSION

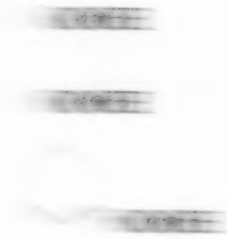
Die Arbeit, die im Grunde an die Arbeit von JANÁK[illegible] über Identifizierung organischer Stoffe mittels dieser Pyrolyse anschliesst, umfasst im Ganzen die Spaltung von 50 aromatischen Stoffen. Die Spaltung wurde unter solchen Bedingungen durchgeführt, dass es zur Spaltung des aromatischen Kernes kam, so dass die Spaltprodukte verhältnismässig einfache gasförmige Substanzen waren.

Durch Verarbeitung der Tabellen I–IV, in denen die einzelnen gespaltenen Substanzen angeführt sind, erhielten wir die Tabelle V, aus der der Anteil der einzelnen Funktionsgruppen des aromatischen Kerns an der Entstehung der Spaltprodukte ersichtlich ist. Z.B. kann man aus dieser Tabelle ablesen, dass durch Spaltung von Carbonsäuren immer nur Wasserstoff und Kohlendioxyd entsteht, aus aromatischen Nitrosubstanzen nur Wasserstoff und Kohlenoxyd. Falls sich am aromatischen Kern mehrere verschiedene Funktionsgruppen befinden, summiert sich der Einfluss der einzelnen Funktionsgruppen. Selbstverständlich gibt es auch Ausnahmen von dieser Regel, die in der Bemerkung zur anschliessenden Tabelle enthalten sind.

Wenn man auch aus dieser Tabelle nicht den Stoff genau identifizieren kann, lässt der Befund doch auf charakteristische Eigenschaften schliessen, allenfalls welcher Stoffgruppe die Substanz angehört. Die Tabelle gibt bloss eine grobe Übersicht, eine feinere Aufteilung kann man durch semiquantitative Auswertung des Auftretens der einzelnen pyrolytischen Produkte erreichen, was in den Tabellen

[illegible]

[illegible]

	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
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	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

[illegible]

TABELLE I *Fortsetzung*

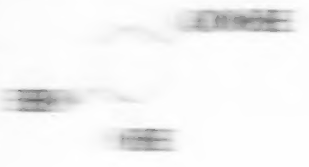
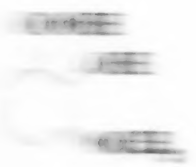
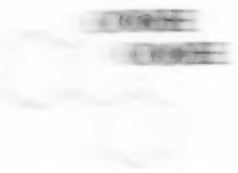
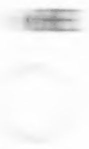
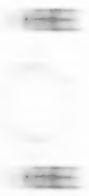
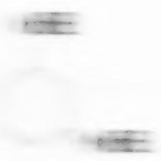
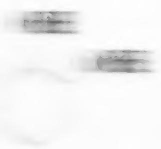
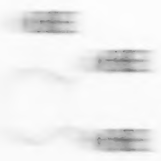
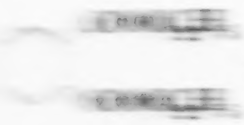
	H_2	CO	CH_4	CO_2
	———	———	—	—
	———	—	——	—
	————	—	—	——

TABELLE II

PYROLYSENPRODUKTE VON PHENOLEN, CARBONSÄUREN UND ESTERN

	H_2	CO	CH_4	CO_2
	————	—	—	—
	————	——	——	—
	————	——	———	—
	————	——	———	—
	——	———	——	—
	————	—	——	—

Fortsetzung S. 402

TABELLE III (*Fortsetzung*)

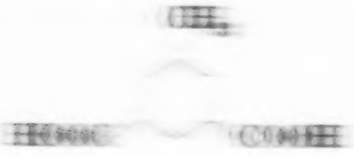
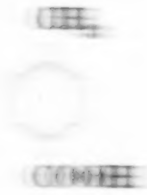
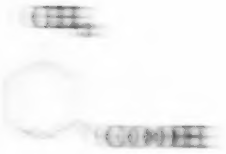
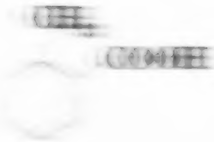
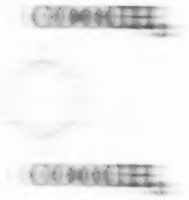
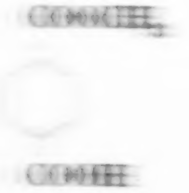
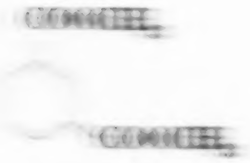
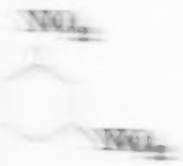
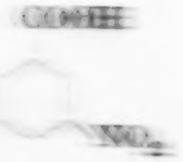
	H_2	CO	CH_4	CO_2
 CH_3 HOOC COOH	++	—	+	++
	+++	Spuren	+	+
	+++	Spuren	+	+
	+++	—	+	+
 $COOCH_3$ $COOCH_3$	+++	+	+++	—
 $COOCH_3$ COOH	+++	+	+	+
 $COOCH_3$ $COOCH_3$	+++	+	+++	+

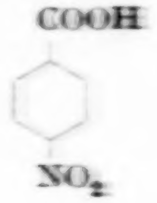
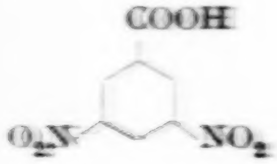
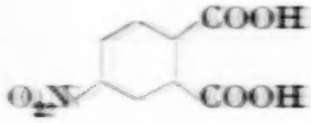
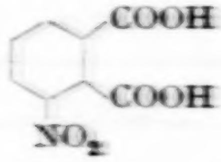
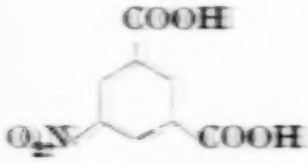
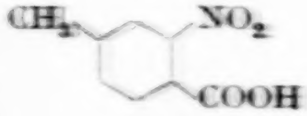
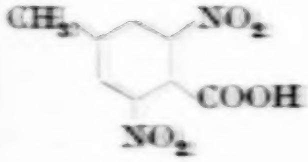
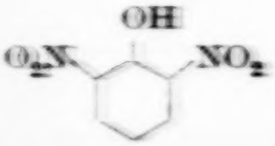
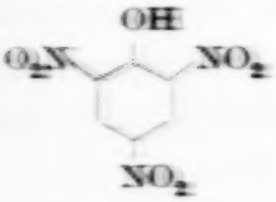
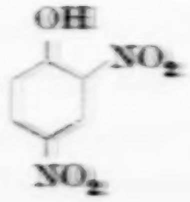
TABELLE IV

PYROLYSENPRODUKTE VON NITROSÄUREN UND NITROPHENOLE

	H_2	CO	CH_4	CO_2
	+++	++	—	—
	+++	+	—	+

(Fortsetzung S. 403)

TABELLE III (*Fortsetzung*)

	H_2	CO	CH_4	CO_2
	+++	+	—	+
	++	++	—	+
	++	+	++	++
	++	+	—	++
	++	+	—	++
	++	+	+	+
	++	++	+	+
	+	++	—	+
	+	+++	—	+
	+	++	—	—

(*Fortsetzung S. 404*)

TABELLE III (*Fortsetzung*)

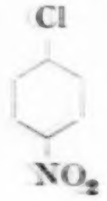
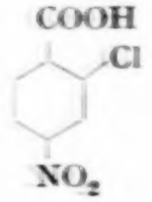
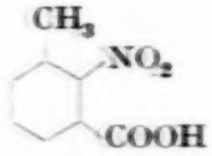
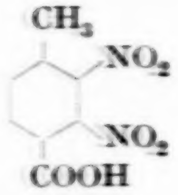
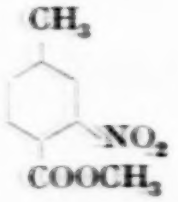
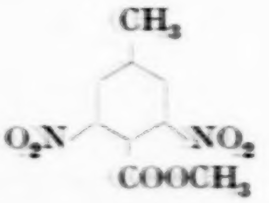
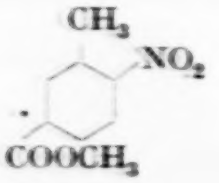
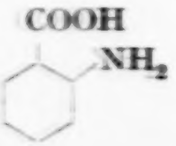
	H_2	CO	CH_4	CO_2
	++	+	+++	—
	++	+	+++	+

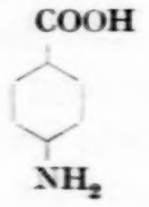
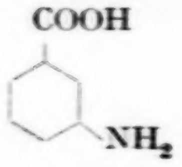
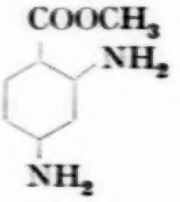
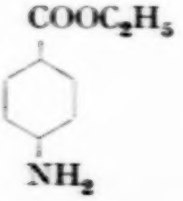
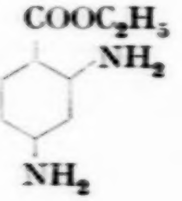
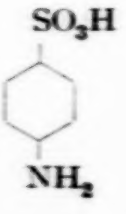
TABELLE IV

PYROLYSENPRODUKTE VON VERSCHIEDENSTEN AROMATISCHEN VERBINDUNGEN

	H_2	CO	CH_4	CO_2
 CH₃ NO₂ COOH	++	+	—	+
 CH₃ NO₂ NO₂ COOH	+	+++	—	+
 CH₃ NO₂ COOCH₃	++	++	++	—
 CH₃ O₂N NO₂ COOCH₃	+	++	+	+
 CH₃ NO₂ COOCH₃	++	++	+	—
	++	—	—	+

(Fortsetzung S. 405)

TABELLE IV (*Fortsetzung*)

	H_2	CO	CH_4	CO_2
	++	—	—	+
	++	—	—	+
	++	—	+	+
	++	—	—	+
	++	—	—	+
	++	—	—	—

durch die Anzahl von Kreuzen bezeichnet wird. Z.B. bei Mono-, Di-, und Trihydroxybenzolen steigt die Kohlenoxydmenge (im Vergleich zu anderen Bestandteilen) mit steigender Anzahl von Hydroxylgruppen. Dieselbe Regel gilt ebenfalls bezüglich des entstandenen CO_2, in Hinsicht auf die Anzahl von Carboxygruppen im Molekül.

Es ist auch nötig zu vermerken, dass im Laufe der pyrolytischen Spaltung manchmal Stoffe von längerer Retentionszeit auftreten als CO_2 (z.B. C_2H_6 bei der Spaltung von Äthylestern), welche aber nicht in Betracht gezogen wurden, da ihre Entfernung aus der Kolonne höhere Temperaturen erfordert, was den ganzen Vorgang verlängern würde. Diese Bestandteile bleiben also in der Kolonne adsorbiert und nach Beendigung des Arbeitscyklus können sie durch Erwärmen aus der Kolonne entfernt werden. Durch die Pyrolyse entstehen auch immer Kondensationsprodukte, welche noch weniger flüchtig sind, als der der Pyrolyse unterworfene Stoff und gewöhnlich an den Wänden des Eintrittsendes der Kolonne abgeschieden werden. Es ist möglich sie durch Auswischen mit einem Wattebausch aufzufangen und aufzulösen und allenfalls weiter zu identifizieren (z.B. auf papierchromatographischem Wege).

Die Pyrolyse von Nitrosubstanzen zeigt die Eigentümlichkeit, dass die Substanzen früher sublimieren, ehe es zum Zerfall kommt. Dem Zerfall unterliegt nur ein kleiner Anteil der Moleküle und deshalb ist es für das chromatographische Spektrum der Nitrostoffe charakteristisch, dass es nur geringe Stufenhöhen aufweist, deren gegenseitige Beziehungen aber der Anzahl der Substituenten entsprechen, ebenso wie es bei den Spektren anderer Stoffe der Fall ist (siehe Fig. 3).

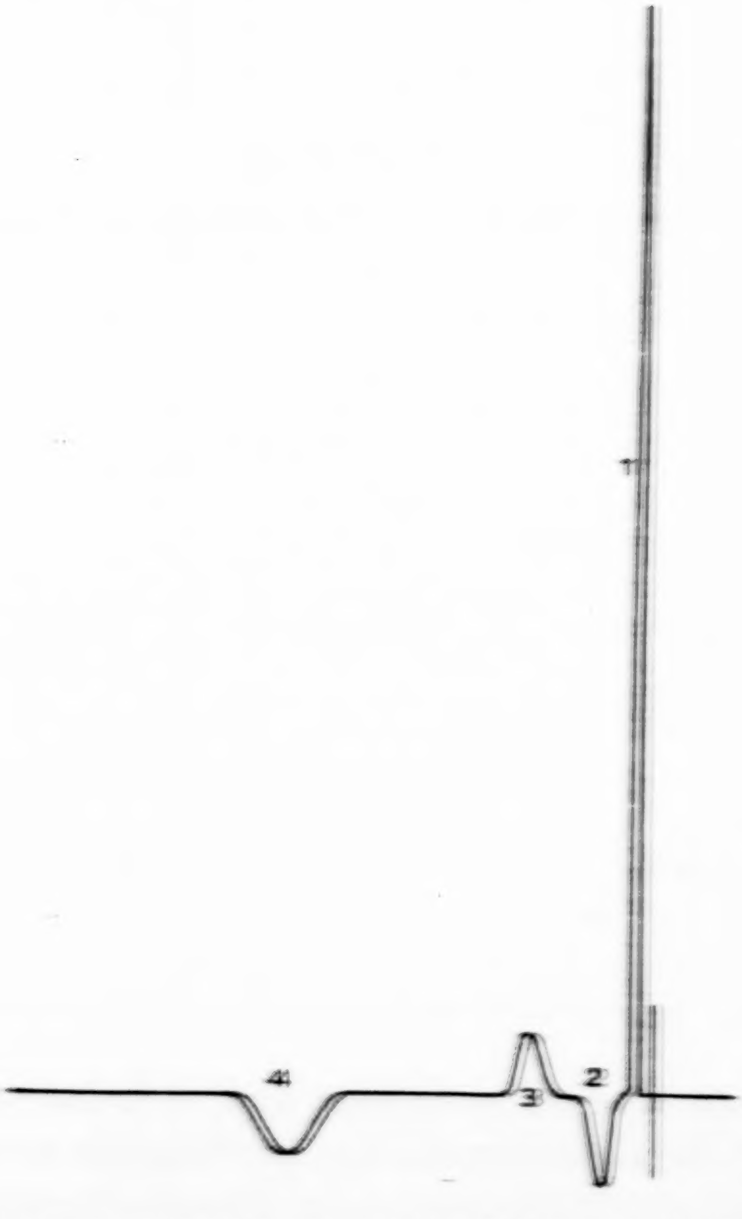


Fig. 3. 1. Wasserstoff; 2. Kohlenoxyd; 3. Methan; 4. Kohlendioxyd.

Zeigt sich bei einem Stoff eine auffällig geringe Menge von Wasserstoff, so zeugt das für gewöhnlich von einer grösseren Anzahl von Substituenten am aromatischen Kern.

Es ist selbstverständlich, dass der Verlauf der pyrolytischen Spaltung ohne Zusatz eines Katalysators ein anderer ist, als mit Zusatz (siehe Tabelle V). Weiter werden sich auch Unterschiede bei Verwendung verschiedener Katalysatoren zeigen, so dass man die Identifizierung noch genauer durchführen kann.

TABELLE V

PYROLYTISCHE ZERSETZUNG MIT DEM KATALYSATOR ($CoCl_2$)

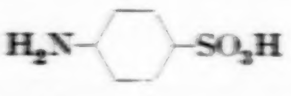
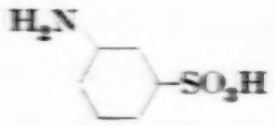
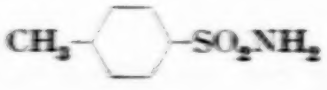
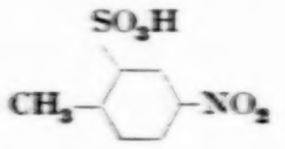
	H_2	CO	CH_4	CO_2
	+ +	+	—	+
	+ +	+	—	—
	+ +	+	+	+
	+ +	+	—	+

TABELLE VI

PYROLYTISCHE ZERSETZUNG OHNE KATALYSATOR
DER ANTEIL DER EINZELNEN FUNKTIONSGRUPPEN
DES AROMATISCHEN KERNS AN DER ENTSTEHUNG DER PYROLYSENPRODUKTE

Gruppe	H_2	CO	CH_4	CO_2
–COOH	+	—	—*	+
–OH	+	+	+	—**
$-NO_2$	+	+	—	—
$-CH_3$	+	—	+***	—
$-COOCH_3$	+	+	+	±
$-COOC_2H_5$	+	+	+	±
–Cl	+	—	+	—
$-NH_2$	+	—	—	—
$-SO_3H$	+	—	—	—

* Ausser *o*-Stellung bei den Carbonsäuren.
** 2,6-Dinitrophenole geben auch CO_2.
*** Bei *o*-Stellung $-CH_3$ und Gruppe die Sauerstoff enthält, erscheint CH_4 nicht.

Wir haben versucht zwecks Information den Einfluss eines Zusatzes von $CoCl_2$ (Kobalt(II)chlorid) festzustellen und es hat sich z.B. gezeigt, dass Stoffe, die ohne Katalysator nur Wasserstoff anzeigen, bei Verwendung von Co eine deutliche Stufe von Kohlenoxyd (allenfalls auch von Kohlendioxyd) aufweisen.

Wie aus der Arbeit hervorgeht, wurde vorläufig nur eine kleine Anzahl von Faktoren, die die pyrolytische Spaltung beeinflussen geprüft. Der Zweck der Arbeit war aber auf neue Möglichkeiten einer Identifizierung fester organischer Stoffe hinzuweisen. Wenn dieser Vorgang auch keinesfalls die bisherigen Methoden der Identifizierung ersetzen kann, wird sie doch in vielen Fällen eine geeignete Ergänzung darstellen.

ZUSAMMENFASSUNG

Die Arbeit befasst sich mit der Identifizierung fester nichtflüchtiger organischer Stoffe unter Benützung der Gaschromatographie. Im Prinzip beruht das Verfahren auf der pyrolytischen Spaltung dieser Stoffe und der chromatographischen Analyse der Spaltprodukte die durch Erwärmung der festen Stoffe unter reproduzierbaren Bedingungen entstehen.

Sie befasst sich mit der Beziehung zwischen der Bildung der einzelnen pyrolytischen Produkte und fallweise ihrer Menge und ihrer Struktur.

SUMMARY

The identification of non-volatile organic solids by means of gas chromatography is discussed. In principle, the proposed method consists in a pyrolysis of the substances and a chromatographic analysis of the degradation products obtained under reproducible conditions.

The relation between the formation of the various products and their respective quantities and structure is discussed.

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A METHOD OF LIPOPROTEIN ELECTROPHORESIS

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(Received January 23rd, 1961)

The analysis of serum lipoproteins has been shown to be important for numerous clinical purposes[1,2]. Despite its clinical value, however, lipoproteins are still not widely determined for clinical purposes on a routine basis. Since limitations of methods may be partly responsible, further methodological developments could be of much value in this field.

Filter paper electrophoresis is a relatively simple method for lipoprotein determinations, but has the disadvantage that some lipoproteins are highly adsorbed by the filter paper. This results in tailing and errors in quantitation as well as changes in relative mobility of the adsorbed fractions[3].

The electrophoretic method utilizing a semi-fluid or thixotropic buffer solution as medium appeared suitable for lipoproteins, since adsorption effects are largely avoided[4]. The procedure can be conducted in an apparatus which combines electrode compartments and the running surface in a single vessel. This reduces the preparations necessary for the analysis of a number of samples, primarily, to pouring a stabilized solution in the vessel[5].

Staining the lipoproteins in the serum before the separation, as described by McDONALD AND RIBEIRO[6], permits them to be followed during the migration, and to be quantitated without errors due to rinsing excess stain from the background. In order to separate enough serum to obtain accurate optical density values, however, it was found necessary to pour the stabilized buffer, not as a thin film, but as a solution 1/4 inch thick. A larger quantity of serum could be applied with the thicker solution[7]. The electrophoresis of lipoproteins by this procedure was investigated. The experimental methods used and the results obtained are described below.

MATERIALS AND METHODS

Pre-staining of serum lipoproteins

A saturated solution of Sudan Black B in ethylene glycol was prepared according to the method of McDONALD AND RIBEIRO[6]. This solution was stable at room temperature. One volume of the Sudan Black B solution was added to 2 volumes of serum, at least 1 day before the electrophoretic separation. The serum was stored in a refrigerator at 3°.

Electrophoretic method

The electrophoresis was conducted as reported previously, except for the modifications described below. The apparatus can easily be constructed of plexiglas or other inert material. A number of 1/4 in. thick glass strips were positioned on the level, vinyl-covered platform so as to produce a series of parallel migration paths (Fig. 2). A volume of 0.05 M veronal buffer, pH 8.6, sufficient to fill the electrophoretic vessel, was prepared, and enough Difco Purified agar was weighed out to make a 0.16% solution in this volume. (Difco Bacto agar cannot be substituted for this procedure, as will be discussed later.) The agar was heated to an active boil in about 1/10 of the buffer and was immediately poured back into and mixed with the remaining 9/10 of the buffer. The buffer was poured into the electrophoretic vessel to a depth of 1/4 in. over the platform, level with the tops of the glass strips. In order for the solution to acquire sufficient rigidity to control convection, it was permitted to stand for 30 min without any current (this wait is not necessary when the buffer is layered as a thin film).

At the end of this period, the temperature of the solution should be between 25–30°. If it is higher, the agar may have been boiled in too large a portion of the buffer. A potential gradient of 4 V/cm was applied. A strip of Whatman No. 17 filter paper, 3/16 in. wide, was dipped in each of the prestained sera and blotted briefly. Each strip was placed with a forceps in the buffer solution with its width in the vertical plane, and its length perpendicular to the direction of the current, as shown in Fig. 2. The strips should not protrude above the surface, and will remain in position when released. The Whatman No. 17 paper retains about 0.5 ml of serum per sq. in., and the volume of serum to be separated can be varied according to the length of the strip.

The samples were permitted to migrate until a distinct separation between α_2- and β-lipoproteins was obtained, which required about 3 hours. The power source was turned off, and each lipoprotein band was lifted between two aluminum sheets and inserted into a 10 ml graduated test tube. This operation is illustrated in Fig. 1. The two sheets are pushed down vertically into the solution, one on each side of the band to be isolated. They are brought together at the bottom, and lifted up to the mouth of the tube. A bottom corner of the two sheets is inserted into the mouth. Separation of the sheets then permits the band to fall into the tube. Any remaining drops may be wiped into the mouth of the tube.

Each of the tubes containing the lipoprotein bands was diluted to 4.5 ml with the ethylene glycol. Drops on the side of the tube can be washed down with the diluent. The tubes were mixed and placed in a hot water bath for about 5 min. After the blue particles dissolved to form a clear solution, the optical density was determined at 590 mμ in a Beckman D.U. spectrophotometer. The per cent of each lipoprotein was calculated from the ratio of its optical density to the total optical density of all three bands.

As an alternative method of quantitation, the lipoproteins could also be measured by direct photometry by a procedure previously reported[8]. Wavelengths of both

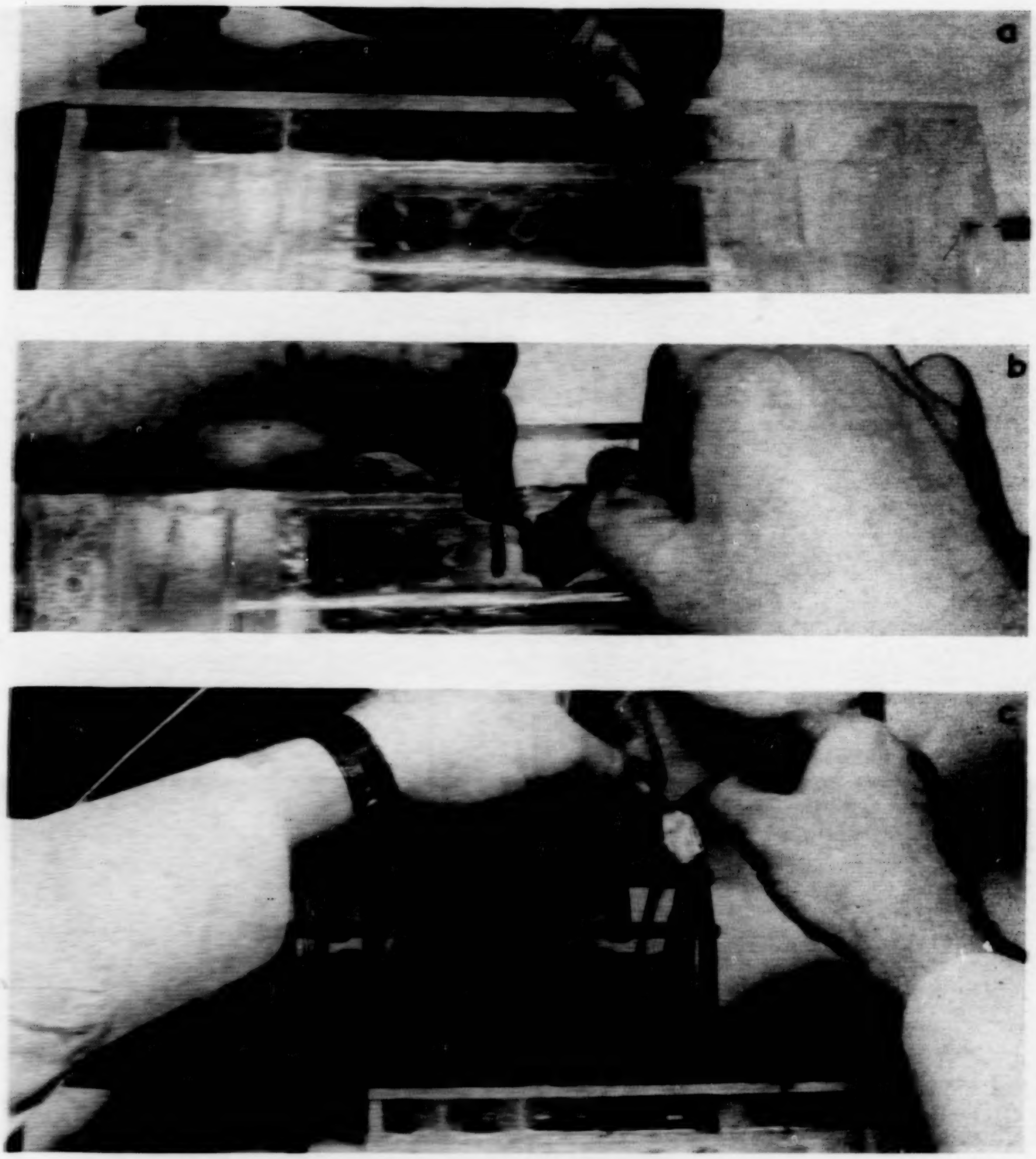

Fig. 1. Transfer of lipoprotein band. An aluminum sheet is inserted down on one side of the band in (a), and another one is inserted on the other side in (b). They are brought together at the bottom and lifted over a graduated test tube in (c). The sheets are parted, releasing the band into the tube.

590 and 200 mμ were used in order to detect both lipid stain and total serum proteins. The buffer used for this purpose contained 0.066 M of boric acid and 0.027 M of LiCl per l, and was adjusted to a pH of 8.6 with NaOH.

RESULTS

An estimate of the reproducibility of the method of removing each band and measuring its optical density was obtained by analyzing 8 samples of the same serum. The results are shown in Table I.

In order to determine whether this reproducibility is adequate for clinical pur-

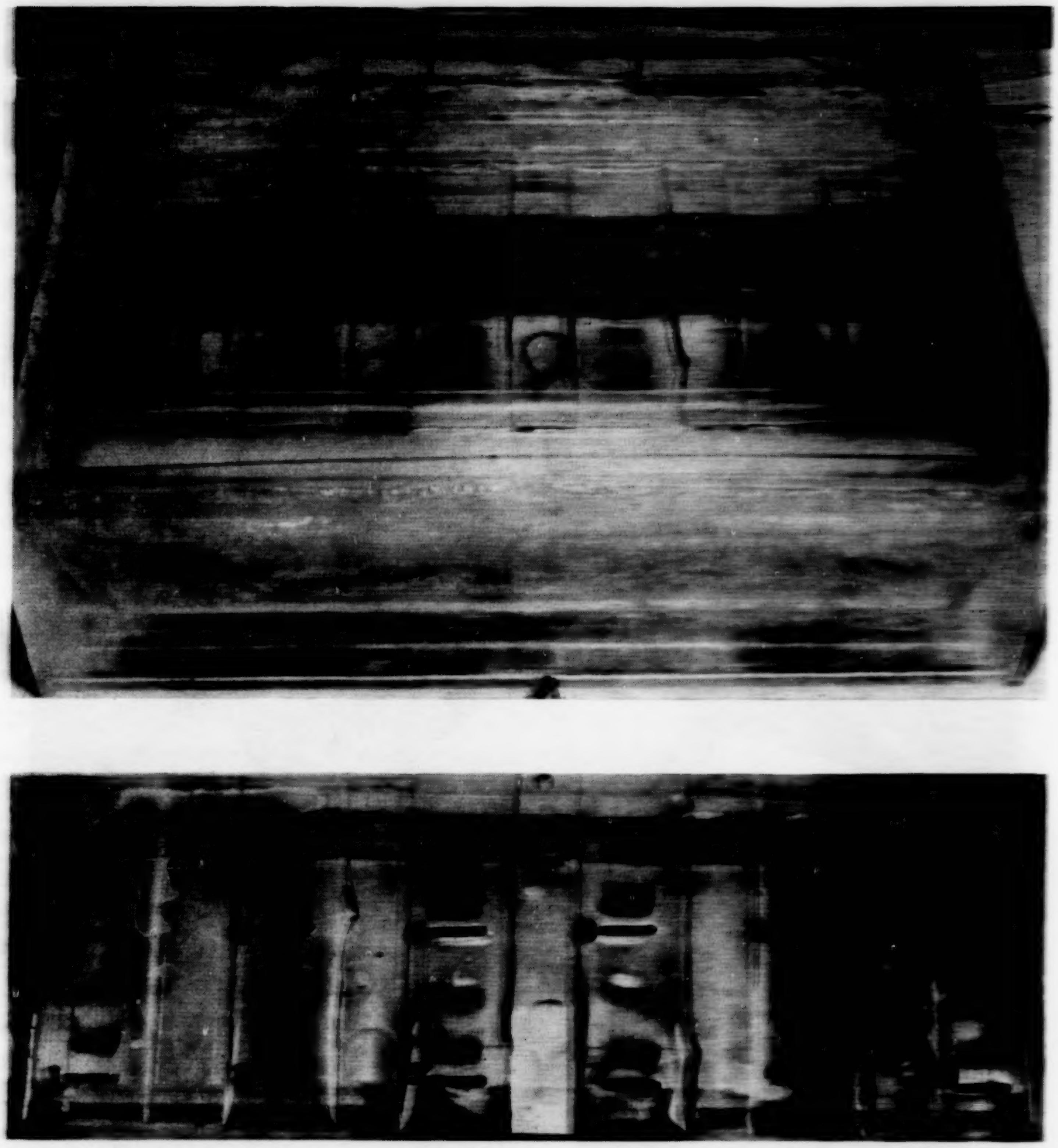

Fig. 2. Lipoprotein analysis of 12 sera. Entire apparatus is shown above, close up of sera is shown below.

TABLE I

REPRODUCIBILITY OF MULTIPLE LIPOPROTEIN DETERMINATIONS ON A SINGLE SERUM

Sample No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
% β-Lipoprotein	53.3	55.8	53.3	54.4	50.2	52.8	53.0	56.9
% α_2-Lipoprotein	22.5	17.1	18.2	19.8	19.4	21.9	22.9	19.1
% α_1-Lipoprotein	24.2	27.1	28.4	25.9	30.4	25.3	24.2	24.0

poses, the serum lipoproteins were analyzed in 12 fasting individuals, 6 of whom were apparently in good health, and 6 of whom were patients expected to have abnormal lipoprotein patterns. The results are presented in Table II and in Fig. 2. The magnitude

TABLE II

NORMAL AND ABNORMAL SERUM LIPOPROTEIN VALUES

Serum No.	*% β*	*% α_2*	*% α_1*	*Age*	*Sex*	*Clinical description*
1	53.3	22.5	24.2	28	M	apparently in good health
2	51.1	17.3	31.6	27	F	apparently in good health
3	50.8	24.8	24.4	27	M	apparently in good health
4	49.2	21.5	29.3	35	F	apparently in good health
5	45.5	19.7	34.8	32	F	apparently in good health
6	42.8	23.6	33.6	23	F	apparently in good health
7	81.0		19.0	25	F	infectious hepatitis
8	70.0	21.6	8.4	37	F	essential hyperlipemia, myocardial infarction
9	52.3	34.8	12.9	38	M	hyperlipemia, angina pectoris
10	59.2	23.0	17.8	51	M	myxedema
11	59.9	27.9	11.7	69	F	intrahepatic obstructive jaundice secondary to chlorpromazine
12	36.0	22.4	41.6	26	F	sarcoidosis

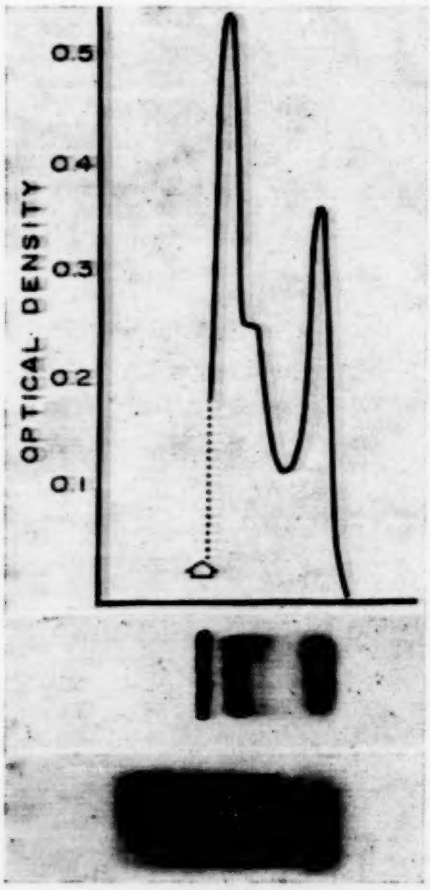

Fig. 3. Photoelectric curve and photographic film at 590 mμ. Bottom film was obtained at 200 mμ.

of the differences between the presumably normal and abnormal values demonstrates that the method is sufficiently reproducible to distinguish them.

Fig. 3 presents the results obtained when the lipoprotein bands were separated and measured directly without removal. A monochrometer light source was positioned above a quartz cell and photographic film or a photocell was used underneath. The volume of serum applied with the Whatman No. 17 paper, in the 1/4 in. thick solution, resulted in suitable optical densities for accurate measurement at 590 mμ. At 200 mμ, however, the optical densities were too great for application of Beer's law.

For purposes of comparing relative mobilities of lipoproteins to other proteins in the same serum, better resolution can be obtained by applying less serum, and reducing the thickness of the buffer solution to a 1.5 mm thick film[8]. This was done and Fig. 4a illustrates the relative mobility of lipoproteins when 0.16 % Difco Bacto agar was substituted for Difco Purified agar. The β-lipoprotein migrated more rapidly, and all the lipoproteins formed a single, diffuse band. This confirms what has been previously reported by WIEME[9]. When 1.5 % potato starch was substituted for the 0.16 % Purified agar, the results shown in Fig. 4b were obtained. The results with starch appear comparable to those obtained by others workers[10].

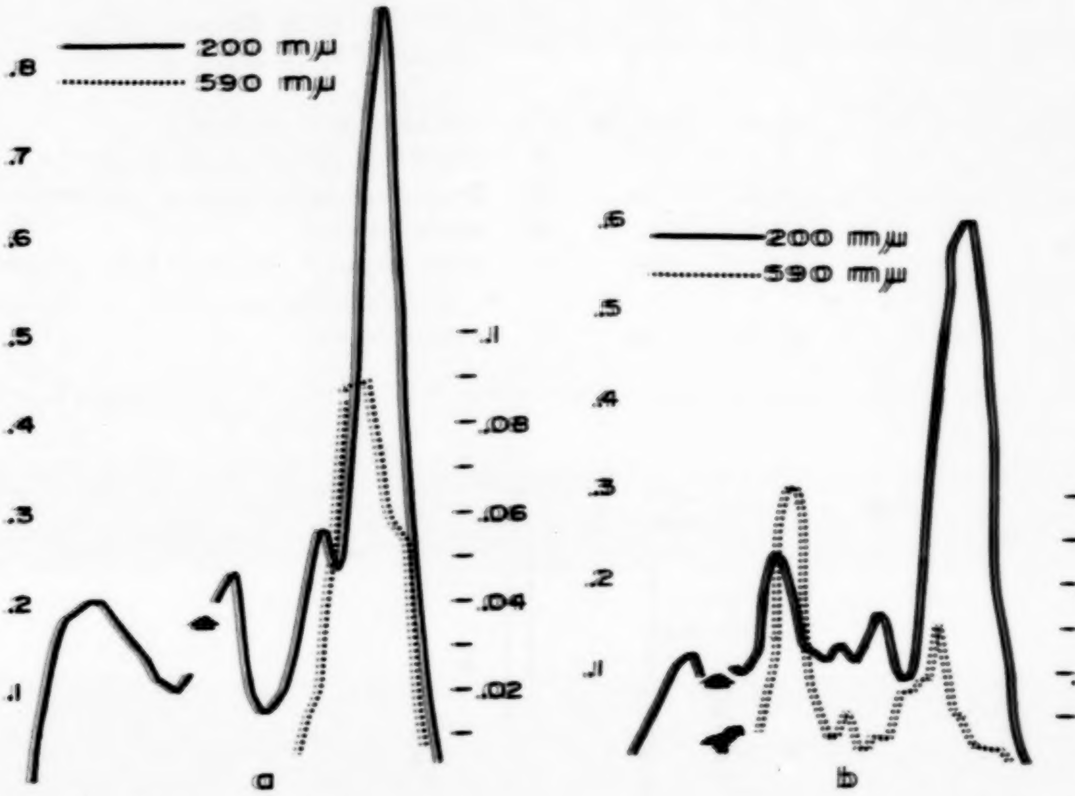

Fig. 4. Optical density distributions obtained with 0.16 % Difco Bacto agar in (a), and with 1.5 % potato starch in (b). Scales at 200 mμ are on the left, and at 590 mμ are on the right.

DISCUSSION

In the present procedure, the lipoprotein bands appear to migrate without significant tailing, since little density can be detected between the β-lipoprotein and the line of application (Fig. 2).

The resolution of the α_2-lipoprotein may be important in view of the investigations which indicate that this fraction is elevated in patients with arteriosclerotic diseases[11,12].

The only serum shown in Fig. 2 which did not reveal a separation between α_2- and β-lipoproteins was from a patient with infectious hepatitis (No. 7 in Table II and Fig. 2). Since atypical lipoproteins have been found in individuals with this disease[13], the lack of separation may be due to the production of heterogenous lipoproteins of intermediate mobility.

SUMMARY

An electrophoretic method is described for the routine analysis of lipoproteins. Use of a semi-fluid medium for the migration minimizes adsorption of lipoproteins by the medium. α_1-, α_2-, and β-Lipoproteins are separated by this procedure. Little working time per sample is required when a number of sera are analyzed together.

The lipoprotein bands can be quantitated by isolation and spectrophotometric measurement. It is also possible to quantitate the bands by direct photometry. The use of light at wavelengths of both 200 and 590 mμ permits comparison of the relative mobilities of the lipoproteins to other proteins in the serum.

The separation allows recovery of the lipoprotein fractions in quantities which may be sufficient for further uses or investigations.

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CHLORANILIC ACID AS A REAGENT IN THE PAPER CHROMATOGRAPHY OF NITROGENOUS COMPOUNDS

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(Received January 19th, 1961)

INTRODUCTION

In previous papers we studied the use of chloranilic acid in the paper chromatography of inorganic compounds[1,2] and in the paper chromatography of the sodium salts of organic acids[3]. In this last case[3] we found that the sensitivity of the reagent towards the sodium salts of nitrogen-containing organic acids, such as nicotinic and *p*-aminobenzoic acids is higher than the values expected.

This was considered to be due to the reaction of chloranilic acid with the nitrogen of the pyridine nucleus, in the first case, and with the aromatic amine, in the second. This assumption was based on the work of BARRETO *et al.*, who used chloranilic acid for the assay of caffeine, theobromine and theophylline[4], coniine[5] and nicotinamide[6].

The present work was carried out in order to determine the sensitivity of chloranilic acid towards different types of nitrogenous compounds, in view of the possibility of applying it as a reagent in the paper chromatography of these substances.

MATERIALS AND METHODS

Reagent

Chloranilic acid was used as a 0.1% (w/v) solution in amyl acetate. In previous work[1-3] ether was used as solvent, but in the present case we found that some of the spots were eluted by this solvent. Better results were obtained with amyl acetate.The reagent is stable when kept in a dark bottle.

Sample solutions

The samples were dissolved in water or in a suitable organic solvent, in concentrations of 0.1% w/v (except for the alkaloids, when the concentration was 1% w/v).

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Evaluation of sensitivity

As described previously[1], the sensitivity was first evaluated by spot tests on filter paper, followed by the determination of the minimum amount of material identifiable after the chromatographic separation.

Paper chromatography

The ascending technique was employed, using Macherey-Nagel No. 261 filter paper and a solvent system appropriate for the case under consideration.

Detection of the spots

The paper chromatograms were thoroughly dried in the oven at 120° and developed by dipping for 30 sec in an enamel tray containing the reagent (which was afterwards returned to the bottle). The chromatograms were then washed three times with amyl acetate, dried under the hood with the aid of an infra-red lamp and observed by ultra-violet light.

RESULTS

The sensitivity of chloranilic acid towards miscellaneous nitrogenous compounds separated by paper chromatography is shown in Table I, together with the R_F values found with each solvent system. It is worth noting that these values are not necessarily identical with the ones quoted in the literature, since the use of a different brand of filter paper can lead to results that differ, even when the same chromatographic technique is used.

TABLE I

SENSITIVITY (in μg) OF CHLORANILIC ACID TOWARDS VARIOUS TYPES OF NITROGENOUS COMPOUNDS SEPARATED BY PAPER CHROMATOGRAPHY

Compound	*Solvent system*	R_F	*Sensitivity*	*Ref.*
Glycine	BuOH–HAc–H_2O	0.30	2	
Alanine	(40:10:50)	0.43	2	7
Asparagine		0.22	2	
Nicotinic acid	BuOH–acetone–H_2O	0.62	6	
Nicotinamide	(45:5:50)	0.75	2	8
Isoniazid*		0.91	2	
Creatine	BuOH–EtOH–H_2O	0.18	6	9
Creatinine	(80:20:20)	0.40	6	
PABA**	BuOH–NH_4OH–H_2O	0.65	2	10
Sulphanilamide	(40:10:30)	0.60	2	
Caffeine	BuOH–HAc–H_2O	0.15	10	
Theophylline	(100:4:sat.)	0.18	10	11
Theobromine		0.05	50	

* Isonicotinic acid hydrazide.
** *p*-Aminobenzoic acid.

The sensitivity was expressed as the minimum amount (in μg) of the sample easily discernible on the developed chromatogram.

From the table it can be seen that chloranilic acid was tested as a reagent for various types of nitrogenous compounds, such as aliphatic and aromatic amines, amides, and guanidine, pyridine and purine derivatives. In most cases the sensitivity was 2 to 6 μg, but it was lower for the purine derivatives (10 to 50 μg).

DISCUSSION

The three characteristics of a good chromatographic reagent are (a) a high sensitivity, (b) a low specificity and (c) a simple developing technique. As regards the last two conditions, chloranilic acid is a very satisfactory reagent for nitrogenous compounds. Its sensitivity, however, is in general identical with that of the usual developers.

In the case of amino acids the sensitivity is ten to twenty times lower than that of ninhydrin, with the exception of asparagine, when it is of the same order[12]. For pyridine derivatives the sensitivity is very similar to that of cyanogen bromide[13], the usual reagent for these compounds. With respect to aromatic amines, the sensitivity is 2 to 10 times higher than that obtained with diazotized *p*-nitraniline[14].

Guanidine derivatives are usually identified by means of the Sakaguchi reaction, but this reaction gives no results with creatine and creatinine[15]. In this case the reaction with alkaline ferricyanide nitroprusside[16] is used, the results being comparable with those obtained with chloranilic acid.

For the purine alkaloids, the best reagent at the moment is the one suggested by HASSOM *et al.*[17], the sensitivity of which is higher than that of chloranilic acid with respect to caffeine (10 μg) but lower with respect to theobromine and theophylline (50 μg).

The results discussed in this paper show that chloranilic acid can be considered as a suitable reagent for the paper chromatography of nitrogenous compounds.

ACKNOWLEDGEMENTS

The present work was carried out at the Central Laboratory of Tuberculosis, in collaboration with the Institute of Phthisiology and Pneumology of the University of Brazil and with the Department of Agricultural Chemistry of the National School of Agronomy of the Rural University. One of us (R.C.R.B.) received a grant from the National Research Council.

SUMMARY

The authors studied the use of chloranilic acid as a reagent in the paper chromatography of various nitrogenous compounds, such as aromatic and aliphatic amines, amides, and guanidine, pyridine and purine derivatives.

The sensitivity was found to vary from 2 to 6 μg in most cases, and from 10 to 50 μg for the purine alkaloids.

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CHROMATOGRAPHIC ADSORPTION OF AZOBENZENES AND AROMATIC NITROGEN HETEROCYCLES ON ALUMINA

STUDIES WITH ALUMINA-IMPREGNATED GLASS PAPER

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(Received January 30th, 1961)

ZECHMEISTER[1] suggested that adsorption on a surface occurs through certain atomic groups, known as "anchoring groups" present in the adsorbate molecule. For chromatography of aromatic and conjugated unsaturated hydrocarbons on alumina or on polynitroarene-impregnated silicic acid KLEMM *et al.*[2,3] have proposed that flatwise adsorption of the substrate is preferred. In such case the entire aromatic system would be considered the anchoring group, though it would seem preferable, rather, to speak of the general π-electronic system of the hydrocarbon as an anchoring site. The adsorption process is believed to involve π-complex formation between the hydrocarbon (electron donor or Lewis base) and the adsorbent (electron acceptor or Lewis acid). For alumina as adsorbent, adsorbability of the substrate was found to increase with increasing number of double bonds, approach to coplanarity, symmetry number, extent of conjugation, and number of sterically unhindered methyl or alkylene groups. Of special interest here are the observations that *trans*-stilbene is adsorbed more tenaciously than its stereoisomer *cis*-stilbene and that amongst groups of conjugated isomeric polyenes the all-*trans* isomers are adsorbed most tenaciously.

In contrast to the case of the stilbenes, it has been noted by COOK[4] and by ZECHMEISTER *et al.*[5] that *cis*-azobenzene and a number of its derivatives are adsorbed more strongly on alumina than are their respective *trans* isomers. Moreover, for the geometric isomers of 1,4-bis-(phenylazo)-benzene the order of adsorbability is reported to be *cis-trans* > *cis-cis* > *trans-trans*[6]. Since molecular models indicate that corresponding geometric isomers involving the azo –N=N– and the vinylene –CH=CH– linkages maintain closely similar spatial geometries, it would seem that the noncoplanar azo compounds contain an anchoring site which fosters adsorbability on alumina to a considerably greater extent than does that of the conjugated π-electronic system. We report here some additional studies between adsorbabilities in the azobenzene–stilbene series and the arene-nitrogen heterocycle series, as well as an interpretation of differences observed for these analogous compounds.

EXPERIMENTAL

Most polynuclear aromatic hydrocarbons used were available in pure form from previous studies[2]. Other substrates were obtained from commercial sources and, if solid, were recrystallized to constant m.p. Quinoline was purified by vacuum distillation. *cis*-Stilbene (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was used without further purification. Chromatography on alumina columns was conducted essentially as in previous studies[2]. In many cases, however, analysis of the residues from evaporation of the effluent fractions was performed spectrophotometrically rather than by m.p. determination. Data on these experiments are presented in Table I.

Chromatography on alumina-impregnated glass paper

A sheet (26 × 56 cm) of Schleicher and Schüll (Keene, N. H., U.S.A.) No. 26 pyrex fiberglass paper was clamped rigidly into a stainless steel frame and dipped into a 15% aqueous solution of reagent grade $Al_2(SO_4)_3$ where it was left for 1 h. It was withdrawn, allowed to drain for 10 min, dipped into 10% NH_4OH for 20 min, and drained for 10 min more. A second and a third dipping (10 min in each solution, 10 min for drainage between solutions) was also made in order to give a more nearly uniform layer of aluminum hydroxide of appropriate thickness. Immersion and withdrawal of the paper was made slowly so as to prevent tearing it. After impregnation of several sheets of paper, concentrations of the impregnant solutions were checked by titration[7]. The impregnated paper was washed by immersion in water for 15–30 min and then dried at 60° for at least 2 h (until dry to the touch). The paper (handled only with clean rubber surgical gloves) was removed from the frame and heated at 250–290° for at least 4 h. The hot paper was trimmed to about 20 × 48 cm and stored flat on a sheet of aluminum in a dry box (containing anhydrous $CaCl_2$) until cool. Just before use it was removed from the box, ruled off lengthwise in centimeters (by means of a lead pencil) and with the zero line about 15 cm from one end and reheated at 290° for 10 min. Small spots (2 μl) of $1–2 \cdot 10^{-2}$ *M* solutions of strongly fluorescent compounds in volatile solvents (ethanol, benzene, or ethyl acetate) were placed 2.5–4 cm apart on the zero line. The solvents were evaporated rapidly by means of a blast of hot air. The paper was transferred to a closed pyrex jar arranged for descending chromatography where it was conditioned in vapors of the developer (anhydrous benzene, reagent grade cyclohexane or a benzene–isooctane mixture) for 12–24 h. To prevent cracking of the brittle impregnated paper it was draped over a 3.5 cm (diameter) glass tube inserted adjacent to the solvent trough. Development of the chromatogram was determined as a function of time using direct observation against a daylight or incandescently lighted background for locating the solvent front and fluorescence in ultraviolet light (furnished by an external 100-W mercury arc source) against a dark background for locating the spots. Runs lasted 1–4 h. Selected linear plots of the centers of the spots (taken as the average of the positions of leading and following boundaries) *versus* the position of the solvent front for three different runs are shown in Figs. 1–3. It should be noted, however, that sets of curves obtained from repetitive

TABLE 1

RELATIVE CHROMATOGRAPHIC ADSORBABILITIES OF SELECTED COMPOUNDS ON ALUMINA COLUMNS

Run	Components used in mixture*		Eluent	Effluent fraction					Extent of separation of components
	name	*m.p. °C*		*No.***	*mg of residue*	*m.p. °C*	*special analytical procedure*	*analytical result*	
1	*cis*-Stilbene	< 25	petrol ether (30–60°)	1	82	< 25	IR (liq. between NaCl plates)	*ca.* pure *cis*-stilbene	good
	trans-Azobenzene	68–69		2	43	64–69	visual observation	yellow	
2	*trans*-Stilbene	123–125	spectral grade iso-octane	1	19	not determined	spectral absorption at 442 mμ of standard (w/v) solutions in iso-octane	pure *trans*-stilbene	
				2	63			71 % *trans*-stilbene	fair
	trans-Azobenzene	68–69		3	32			63 % *trans*-azobenzene	
				6	15			pure *trans*-azobenzene	
3	Anthracene	213–214	petrol ether	1	97	206–210	followed on column by fluorescence		very good
	Acridine	106–110		last	26	96–100	eluted with acetone		
4	Anthracene	211–212	18 % benzene in petrol ether	1	85	207–211			complete
	Phenanthridine	105–106		last	100	103–104.5	eluted with ethanol		

5	Phenanthrene	95–97	petrol ether	1	72	97–99	none		fair
	Phenanthridine	105–106		3	47	104–106			
6	Acridine	108–110	benzene	1	64		IR (20 mg/0.5 ml $CHBr_3$)	*ca.* pure acridine	good
	Phenanthridine	105–106		2	40	not determined	ditto	largely acridine	
				3	59		ditto	largely phenanthridine	
7	Acridine	108.5–110.5	benzene	1	47	93–95.5		*ca.* 60 % acridine	poor
	2-Methylacridine	134.5–135.5		3	65	103–114	compared m.p. of effluent fraction with standard m.p.–composition curve	*ca.* 60 % 2-methylacridine	
				5	8	123–128		*ca.* 80 % 2-methylacridine	
8	Naphthalene	78–80	petrol ether	1	86	78–80			
	Quinoline	< 25		last	94	< 25	eluted with acetone	odor of quinoline	complete
9	Dibenzothiophene	97–98	petrol ether	1	fair size	86–95	IR (nujol mull)	*ca.* pure dibenzothiophene	fair
	Phenanthrene	95–97		5	ditto	90–95	ditto, eluted with acetone	*ca.* pure phenanthrene	
10	Dibenzofuran	83.5–84.5	petrol ether	1	fair size	83.5–84.5			
				middle	very small	90–95	none		very good
	Dibenzothiophene	98–99		last	fair size	97–99			

[*] For each run the components are listed in order of appearance in the effluent. 100 mg of each component.
[**] Numbered in order of appearance.

runs were generally neither superimposable nor linear throughout, though each curve of the set had approximately the same position with respect to the other curves. Also curves from two different spots of the same compound run on one piece of paper were only approximately the same.

It is taken as a basic assumption that adsorbability decreases with increasing rate of movement of a spot on the paper. On this basis other adsorbability relationships found, but not shown in Figs. 1–3, are 2-methylacridine > acridine > benz(*c*) acridine.

DISCUSSION

In order to check the consistency of adsorbability data obtained from alumina columns and alumina-impregnated glass papers, some compounds were chromatographed by both methods. The results for a group of six hydrocarbons run on paper simultaneously are shown in Fig. 1. A total of six runs made with these same substrates all showed the adsorbability relationships 2-phenylanthracene > 1-phenylanthracene > 9-phenylanthracene in agreement with results previously found on columns[2]. However, in

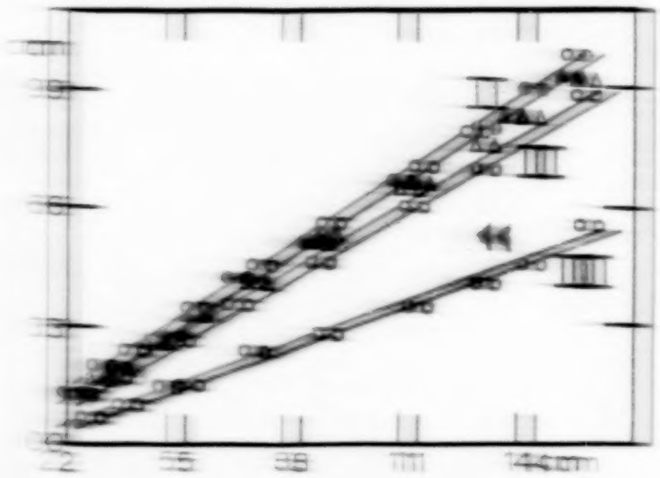


Fig. 1. Vertical axis: distance moved by substrate spot. Horizontal axis: distance moved by solvent front. Solvent: cyclohexane. I: 9-phenylanthracene; II: 1-phenylanthracene; III: 2-phenylanthracene; solid circles: anthracene; solid triangles: 9-methylanthracene; open triangles: pyrene.

non-linear plots the relative positions of anthracene, 9-methylanthracene and pyrene were variable with respect to one another and to 1- and 9-phenylanthracenes (but not to 2-phenylanthracene). It thus appears that the gross differences in adsorbability which were noted on columns were reproduced on paper, but some subtle differences noted in competitive runs on columns[2], *viz.* 9-methylanthracene > anthracene (good separation), pyrene > anthracene (fair separation) and 9-phenylanthracene > anthracene (fair separation), are not consistently detected on the paper. Consistency was found for adsorbability of the substrate pair 2-methylacridine–acridine, former > latter on paper, poor separation on column (*cf.* run 7).

Cyclohexane or isooctane was found to be appropriate for development of the paper chromatogram with aromatic hydrocarbons. Use of benzene as developer was unsatisfactory for these compounds because the rate of movement of the substrates was too large to give sufficient differentiation among them. For the more strongly adsorbed nitrogen heterocycles (Figs. 2 and 3). However, benzene, benzene-isooctane or benzene-cyclohexane was preferable. The nitrogen heterocycles are so strongly adsorbed that it is difficult to elute them from columns with non-polar solvents. For these the use of paper appears to be more satisfactory.

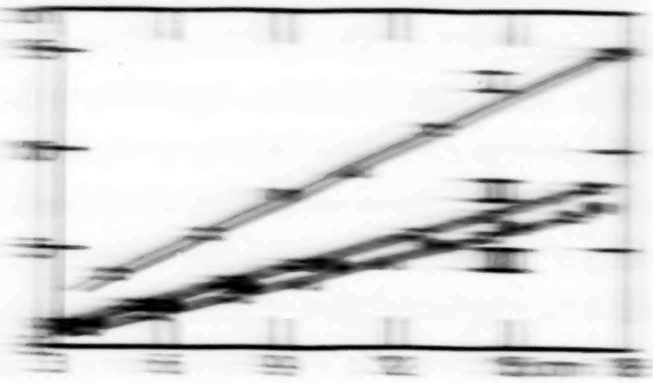


Fig. 2. Vertical axis: distance moved by substance spot. Horizontal axis: distance moved by solvent front. Solvent: benzene. I: [illegible]; II: acridine; III: [illegible].

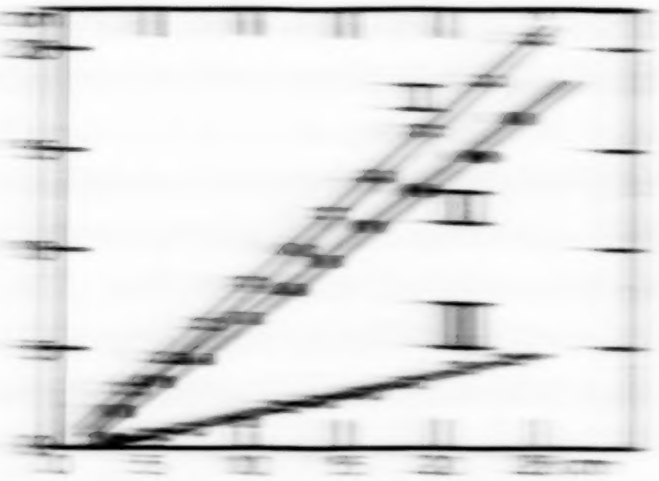


Fig. 3. Vertical axis: distance moved by substance spot. Horizontal axis: distance moved by solvent front. Solvent: benzene-isooctane [illegible]. I: [illegible]; II: [illegible]; III: [illegible].

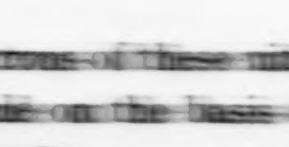
The amount of substance used [illegible] in these chromatograms was sufficient to allow observation of only very strongly fluorescent materials. Thus, attenuation caused the [illegible] spot to become invisible after [illegible], while chrysene could not be followed at all; it formed a spot which fluoresced on the dry paper but did not fluoresce as soon as the solvent front had passed through it. Within the limitations imposed by such characteristics of fluorescence of the adsorbates it is apparent that use of impregnated glass paper offers considerable saving in time and materials over use of columns for correlating adsorbability on alumina with structure of the adsorbate.

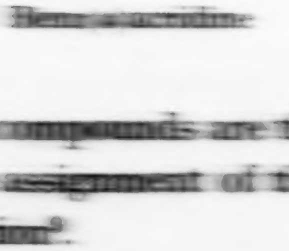
From results in Table I it is apparent that the [illegible] holds for chromatography on alumina [illegible] of —N= and —CH= analogs in the systems *trans*-azobenzene, [illegible] (*cf.* runs [illegible]), phenanthridine, acridine, anthracene (*cf.* runs [illegible]) and [illegible], phenanthrene (*cf.* runs [illegible]). [illegible] studied one finds the orders of adsorbability *cis*-azobenzene > *trans*-azobenzene > *trans*-stilbene > *cis*-stilbene and phenanthridine > acridine > anthracene > phenanthrene > dibenzothiophene > dibenzofuran. Along with the results that quinoline > naphthalene (run 8), and benzo(*f*)quinoline = benz(*a*)acridine = 2-methylacridine > acridine > benz(*c*)acridine > benz(*a*)anthracene, 5,6,11,12-tetraphenylnaphthacene (Figs. 2 and 3, run 7) it is clear that replacement of the =CH— group by an =N— group serves to increase adsorbability and may drastically alter the relations (from those found for hydrocarbons) for the effects of structural features in the molecules on adsorbability. It is, moreover, noteworthy that dibenzothiophene and dibenzofuran, compounds containing the same total number of π-electrons as phenanthridine and acridine plus a similar non-bonding pair of electrons (*n*-electrons) on the hetero-atom, do not exhibit enhancement of adsorbability (as compared to anthracene and phenanthrene).

It is here proposed that adsorption of azo compounds and aromatic nitrogen heterocycles on alumina may occur by (1) *n*-complex formation involving the alumina surface as electron acceptor and the *n*-electrons on the nitrogen atom as the anchoring site, (2) π-complex formation as described previously for polyenes and arenes, or (3) hybrid *n*-π-complex formation involving both *n*-electrons and π-electrons from the adsorbate molecule. At least for the molecules considered in this paper it is believed that *n*-complex formation is energetically preferred so long as steric hindrance to the requisite edgewise or tilted orientation of the adsorbate molecule (with respect to the alumina surface—considered flat) is not too great. Thus, it is presumed that *cis*-azobenzene, quinoline, phenanthridine, acridine, 2-methylacridine, benz(*a*)acridine and benzo(*f*)quinoline form *n*-complexes which are considerably more stable than the π-complexes formed by their hydrocarbon analogs, while *trans*-azobenzene, benz(*c*)acridine, and *trans*-*trans*-1,4-bis-(phenylazo)-benzene form π-complexes which are only slightly more stable than the corresponding complexes formed by their hydrocarbon analogs.

Phenanthridine

Benzo(*f*)quinoline


Acridine


Benz(*a*)acridine

Benz(*c*)acridine

The *n*-electrons of these nitrogen compounds are the most readily polarizable ones in the molecule on the basis of the assignment of the long wavelength electronic absorption band to an *n* → π transition[9].

On the basis of only steric hindrance to *n*-complex formation one would expect

[illegible]

[illegible]

[illegible]

$$[illegible]$$

[illegible]

[illegible]

be interpreted in terms of the formation of π- (from styrene) or *n*- (from the vinylpyridines) complexes of the corresponding $ArCH_2CH_2SH$ intermediates. When Ar is phenyl the flatwise adsorbed intermediate cyclizes readily to benzothiophene. When Ar is 2-pyridyl, there is mainly edgewise adsorption on the nitrogen atom with little resultant formation of thienopyridine but extensive concomitant splitting of the Ar–C bond. For Ar is 4-pyridyl, neither cyclization nor bond splitting is observed.

ACKNOWLEDGEMENT

This research was supported through sponsorship by the United States Air Force, Contract No. AF 49(638)-473 monitored by the AF Office of Scientific Research of the Air Research and Development Command.

SUMMARY

A semi-quantitative method is described for chromatography of fluorescent polynuclear aromatic hydrocarbons and aromatic nitrogen heterocycles on alumina-impregnated glass paper. Combining data obtained from such papers with those from alumina columns it is noted that the isomeric azobenzenes and nitrogen heterocycles are more strongly adsorbed than their hydrocarbon analogs (where CH replaces N). It is proposed that adsorption of such nitrogen compounds occurs preferentially by means of the electron pairs on the nitrogen atoms to form *n*-complexes. If steric hindrance to *n*-complexation is too great the substrate may form a π-complex or a π*n*-hybrid complex instead.

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THE REACTION OF SODIUM OR POTASSIUM NITRITE WITH RUTHENIUM SALTS

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(Received January 11th, 1961)

The paper chromatography and paper electrophoresis of ^{106}Ru tracer has been examined in several publications[1] in relation to the separation of Ru from other fission products. It was observed that Ru tracers separated into several spots owing to the equilibrium mixtures which are formed between the various nitroso-nitrato complexes; the difficulties in identifying these by paper chromatography were shown in the papers by FLETCHER *et al.*[2].

Usually it is now recommended to distill off the ruthenium as RuO_4 before examining the other fission product elements. We have for some time attempted to find a reaction which would yield a single ionic species with ruthenium, irrespective of its valency and complex state, which could then serve as starting point for a paper chromatographic or paper electrophoretic identification. We shall briefly mention some unsuccessful attempts. Thiourea will react with chloro-complexes, but only incompletely with nitroso-complexes; boiling with HBr or oxidation with bichromate in HCl were found to yield single spots when ruthenium chlorides were reacted, but not with nitroso-nitrato complexes, nor with tracer amounts of ruthenium.

The present study was started at the suggestion of Dr. L. M. VENANZI (Oxford), who mentioned to the authors that recent evidence had shown that the compound described as $K_2[Ru(NO_2)_5]$ is actually $K_2[Ru(NO)(NO_2)_4(OH)]$ (see also MURRAY[3]). Thus its preparation does not involve the removal of the nitrosyl group. We decided therefore to investigate the reaction between nitrite and ruthenium nitrates and chlorides, as the problem of removing the nitrosyl group seems to have been the difficulty in our previous attempts to form a single ionic species with ruthenium.

Dr. VENANZI also kindly supplied us with samples of the sodium and potassium salts prepared in his laboratory according to the syntheses given below:

(1) $K_2[Ru(NO)(NO_2)_4(OH)]$ (JOLY AND VÈZES[4]). Potassium nitrite (2 g) was dissolved in water (20 ml) and to the boiling solution, ruthenium trichloride was added in small amounts until a faint precipitate began to appear. A crystal of potassium nitrite was then added to redissolve this and the orange solution evaporated

to about 10 ml. On cooling, the product crystallised out as an orange solid which was purified by recrystallisation from water.

(2) $Na_2[Ru(NO)(NO_2)_4(OH)]$ (JOLY AND LEIDIÉ[5]). Ruthenium trichloride was dissolved in water, and solid sodium nitrite was added in small portions, until the addition of a further portion caused no effervescence. The orange solution was then evaporated until solid began to separate, and the solution left to cool. The product was recrystallised from water.

These two methods do not differ essentially from those quoted in PASCAL[6], and it seems that the same product is obtained irrespective of whether the ruthenium is added to an initial excess of nitrite, or nitrite to an excess of ruthenium.

EXPERIMENTAL

(1) *Preparations and electrophoretic analyses*

All paper electropherograms were carried out with 250 V for 20 min (unless otherwise mentioned) with $N/2$ $NaNO_2$ as electrolyte on paper strips 40 cm long (Papier Arches No. 302), which were sandwiched between glass plates 31 cm long.

We repeated the syntheses mentioned above on a micro-scale, so as to be able to analyse the reaction mixture before crystals are separated.

For the sodium salt, about 50 mg of commercial $RuCl_3$ were dissolved in about 1 ml of water in a 5 ml beaker. Solid $NaNO_2$ was added till the solution, which was kept on the water bath, gave no effervescence. On evaporating, yellow crystals formed, which were filtered off, recrystallised from water and dissolved in about 1 ml of water.

When these three fractions (*i.e.* mother liquor, mother liquor from the recrystallisation and solution of the crystals) are subjected to paper electrophoresis, they all yield a slower orange spot and a faster yellow spot. Both spots react with H_2S to give an intense purple coloration which fades rapidly to a brown stain. The slow orange fraction is most intense in the mother liquor and is only present in traces in the once recrystallised substance. Crystals prepared from the once recrystallised material were found to be the pure yellow fraction.

The potassium salt was prepared by adding commercial $RuCl_3$ in small amounts to 0.4 ml of 10% aqueous KNO_2 on the water bath till a precipitate appears, and then a crystal of KNO_2 was added to redissolve this precipitate. During this operation it was realised that it is rather difficult to assess the exact point at which sufficient $RuCl_3$ had been added. We therefore took another lot of KNO_2 to which we added approximately half the amount of $RuCl_3$ added to the first tube, to ensure that KNO_2 was present in excess. When electrophorised, both solutions, separated into a slower orange and a faster yellow band with mobilities identical to those of the sodium salt.

The samples of Dr. VENANZI were also examined by paper electrophoresis. The sodium salt was found to be the pure yellow compound, while the potassium salt contained some of the orange compound.

Preliminary experiments showed us that a mixture of the orange and the yellow compounds, as formed in the two syntheses, could be converted to an almost pure solution of the yellow compound by simply heating with an excess of $N/2$ $NaNO_2$ on the water bath.

In order to investigate whether a complete conversion is possible, 150 mg of $RuCl_3$ were refluxed with excess $NaNO_2$ (522 mg) in about 14 ml of water (*i.e.* $N/2$ $NaNO_2$) and samples withdrawn at intervals and electrophorised. Already after 5 min refluxing, only a single yellow band could be detected by paper electrophoresis. A similar experiment was carried out by mixing excess KNO_2 with $RuCl_3$. Here two species persisted until the solution was heated for 20 min or more.

Since ruthenium is often present as the nitroso-nitrato complexes in solution, we also investigated if these could be converted to the yellow complex by refluxing with excess $NaNO_2$. A solution of the nitroso-nitrato complexes was prepared by evaporating some crystals of the yellow compound with concentrated nitric acid. The residue was taken up in $N/2$ $NaNO_2$ and yielded initially two anionic fractions. It is only after refluxing for at least 2 hours that only one yellow band is obtained in electrophoretic analysis.

We also carried out the reaction between carrier-free ^{106}Ru (in HNO_3) and $N/2$ $NaNO_2$. When the tracer is evaporated on the water bath and taken up in $NaNO_2$, its electropherogram shows two anionic peaks (Fig. 1). If it is left at room temperature only one peak may be observed after some time, however, with an electrophoretic mobility which is not identical with that of the yellow band. On the other hand, if the solution of the tracer is refluxed for about half an hour, it is converted to a single species which, when mixed with the yellow compound, shows a complete coincidence between the visible band and the activity peak, as shown in Fig. 2. The time of electrophoresis for this experiment was prolonged to 1 hour and the distance moved by the peak was 80 mm.

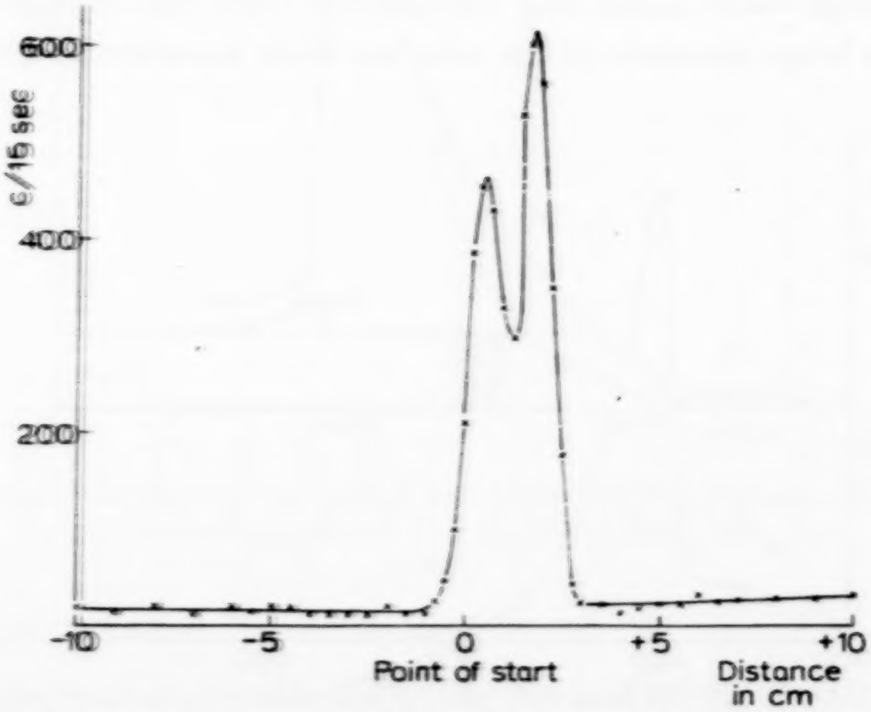

Fig. 1. Distribution of radioactivity on the electropherogram of a mixture of ^{106}Ru tracer and $N/2$ $NaNO_2$ (without heating). The tracer was originally in a nitric acid solution.

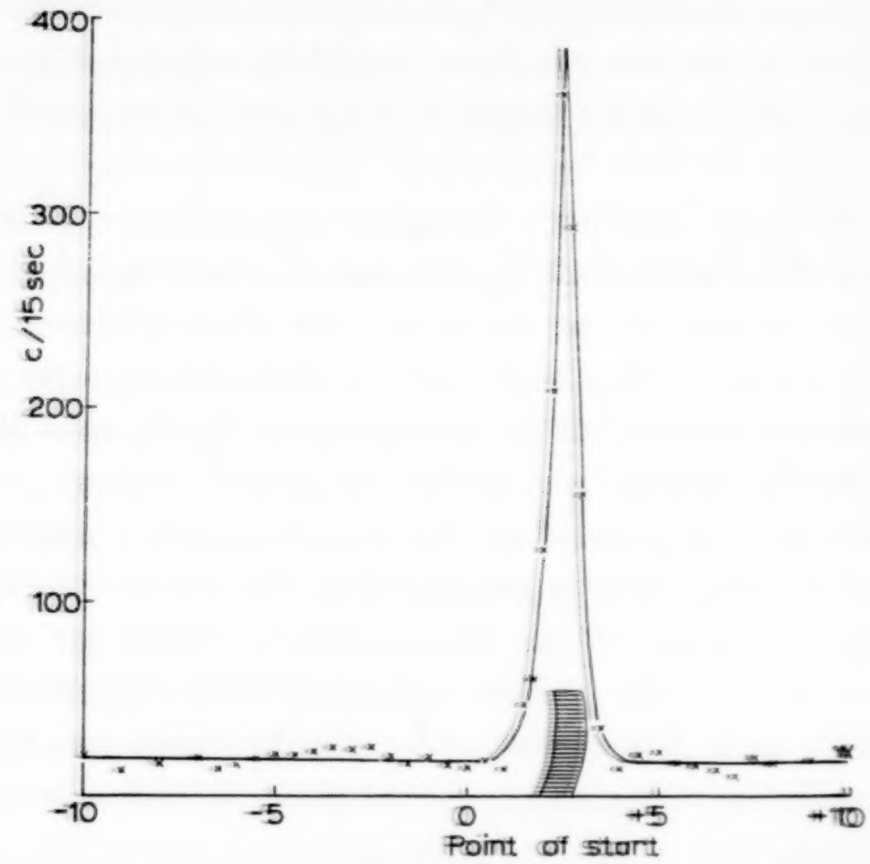

Fig. 2. Electropherogram of a mixture of ^{106}Ru tracer (boiled with $N/2$ $NaNO_2$ for 30 min) and pure $Na_2[Ru(NO)(NO_2)_4(OH)]$.

(2) *Absorption spectra*

In Figs. 3 and 4, the spectra and electropherograms of the sodium and the potassium salt (supplied by Dr. Venanzi) are shown. The spectrum of the yellow band isolated from electropherograms, or obtained by refluxing ruthenium chloride with KNO_2 for 30 min is identical to that of Venanzi's sodium salt, but differs from the impure potassium salt. The difference between the spectra is best shown in Fig. 5 where the yellow band isolated from an electropherogram and the original mixture are superposed.

The slower orange band could only be isolated from electropherograms in small amounts even when large amounts of the mixture were separated on thick (Whatman

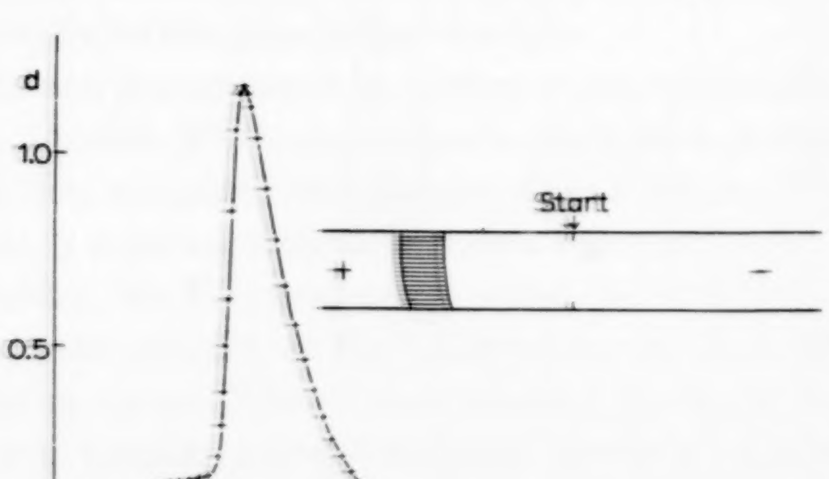

Fig. 3. Spectrum and electropherogram of a sample of $Na_2[Ru(NO)(NO_2)_4(OH)]$ prepared by Dr. Venanzi.

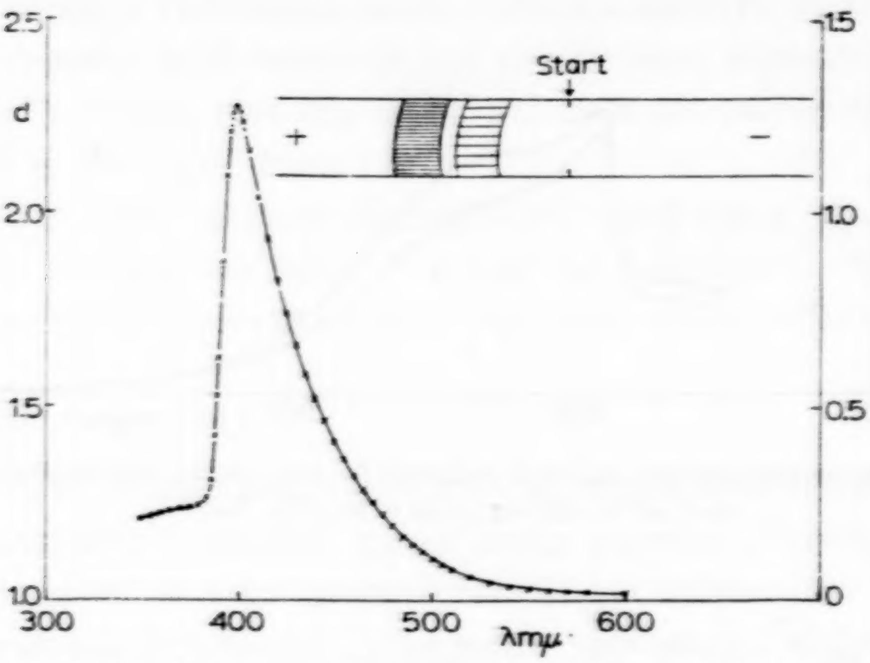

Fig. 4. Spectrum and electropherogram of a sample of $K_2[Ru(NO)(NO_2)_4(OH)]$ prepared by Dr. VENANZI.

No. 3 MM) paper. Fig. 6 shows the spectra of two different extractions as well as the spectrum of a solution of the orange compound aged in $N/2$ $NaNO_2$ for 3 days. The change indicates that some decomposition had taken place. By suitable dilutions of a solution prepared from pure yellow crystals it could be shown that Beer's law is obeyed for the wavelength 398 mμ as shown in Fig. 7.

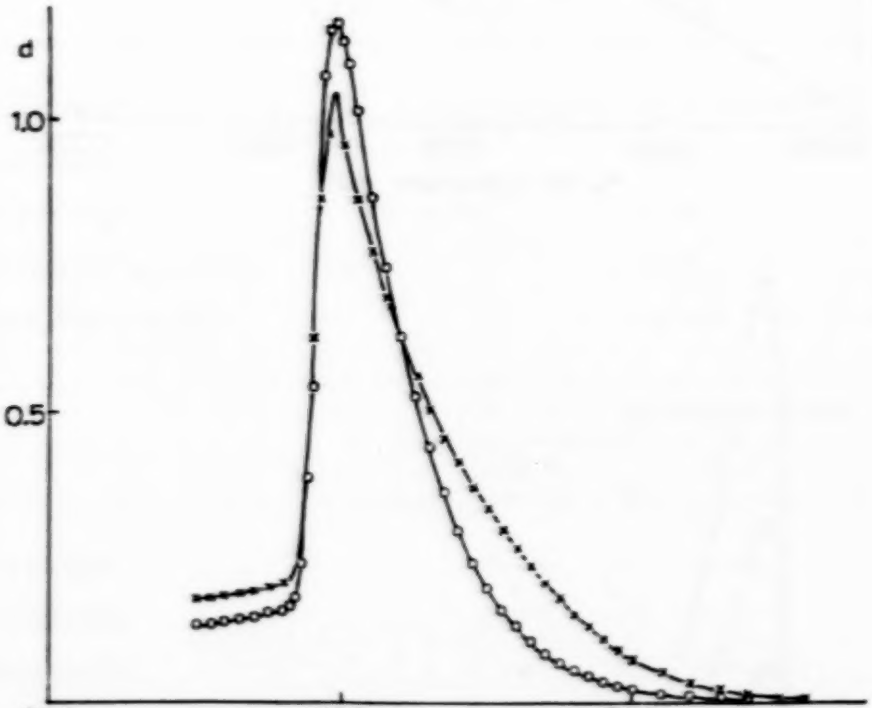

Fig. 5. Superposition of the spectra of a yellow band isolated from a paper electropherogram and an impure sample of $Na_2[Ru(NO)(NO_2)_4(OH)]$.

(3) *Paper chromatography*

The impure products of the syntheses of the Na and of the K salt were examined by paper chromatography.

No adsorption could be noted when they were developed with aqueous $NaNO_2$

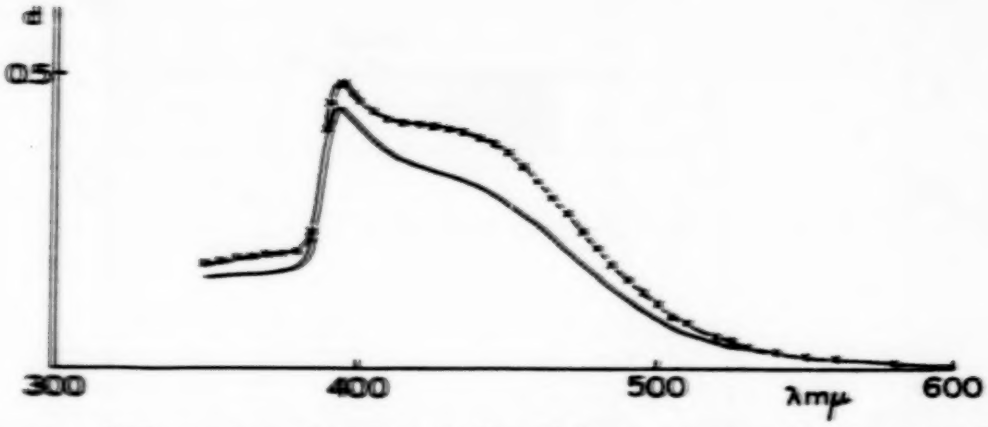

Fig. 6. The spectrum of the orange fraction isolated from a paper electropherogram immediately and after ageing 3 days in *N*/2 $NaNO_2$.

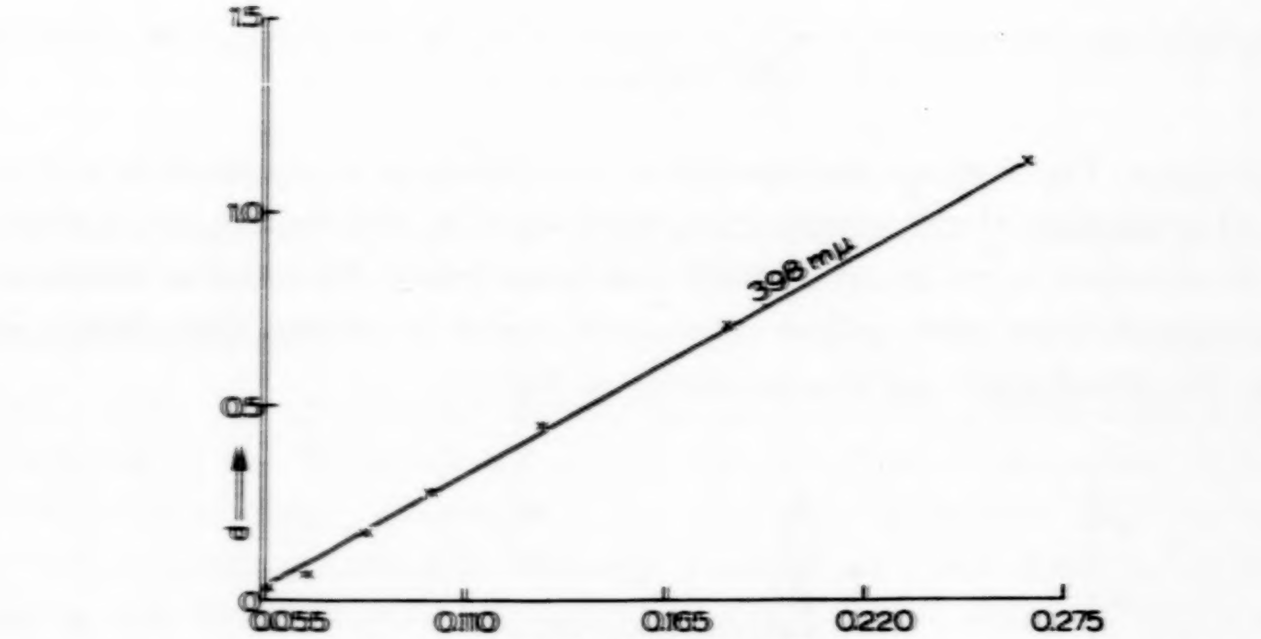

Fig. 7. Verification of Beer's law for the pure yellow $Na_2[Ru(NO)(NO_2)_4(OH)]$.

on Whatman No. 1 paper. In butanol–water there is a spot (R_F *ca.* 0.1) with a forward comet. In butanol–acetic acid–water (5:1:4) the product separates into an orange spot (R_F 0.21) and a yellow spot (R_F 0.28), but both are rather elongated with decomposition trails to the liquid front.

Acetone–water (4:1) and acetone–hydrochloric acid–water (90:5:5) yield two adjacent spots on the acetone front. We had the impression that decomposition occurred during partition chromatography, especially when the solvent is acid.

(4) *Ion exchange chromatography*

Good separations of the orange and the yellow compounds were obtained on Whatman DEAE (diethylaminoethyl-cellulose) paper using aqueous $NaNO_2$ as solvent. The yellow compound moved as a well defined round spot followed by the orange which had a tendency to streak. In 1 *N* $NaNO_2$ the yellow spot moved with an R_F of 0.51–0.53 and the orange with one of 0.27–0.32. Since ion exchange data can often be used for determining the charge on ions[7], we compared the movement of the two compounds with that of ferrocyanide, ferricyanide and nitroprusside using $NaNO_2$ and NaCl of various concentrations as eluants.

TABLE I

R_F VALUES OF SOME ANIONIC Fe AND Ru COMPLEXES ON WHATMAN DEAE PAPER

	Concentration of $NaNO_2$			
	0.1 N	*0.25 N*	*0.5 N*	*1.0 N*
Ferricyanide		0.13 (comet)	0.22 (comet)	0.43
Ferrocyanide		0.4	0.64	0.89
Nitroprusside		0.11	0.26	0.47
$[Ru(NO)(NO_2)_4(OH)]$	0.05		0.33	0.51
Orange Ru complex	0		streak	0.27–0.32

	Concentration of NaCl		
	0.25 N	*0.5 N*	*1.0 N*
Ferricyanide	0.04	0.09	0.25
Ferrocyanide	0.16	0.57	0.85
Nitroprusside	0.09	0.21	0.37

As shown in Table I, the tetravalent ferrocyanide is less adsorbed than the trivalent ferricyanide and the divalent nitroprusside. It seems that such anions show too great a tendency for the formation of ion pairs to make a determination of the charge possible. The observations here recorded seem to be the first separations of such anions on DEAE paper or other modified cellulose paper, and the few results obtained indicate that such separations are of analytical interest.

CONCLUSION

In controlling the reaction between sodium or potassium nitrite and ruthenium salts by paper electrophoretic analysis, it was possible to show that the methods of JOLY AND VÈZES[4] and JOLY AND LEIDIÉ[5] yield a mixture of an orange and a yellow compound. The yellow one is obtained in a pure form if recrystallised twice from water. It is also possible to carry the reaction to completion by refluxing an excess of nitrite with ruthenium chloride or nitrate and controlling the time necessary by paper electrophoresis.

By comparison with analysed samples obtained from Dr. VENANZI it could be established that the spectrum and the electrophoretic mobility of our yellow compound is identical with that of $Na_2[Ru(NO)(NO_2)_4(OH)]$.

The orange by-product was found to be unstable in nitrite solution and is most likely a complex with some unconverted OH or Cl groups or perhaps a polymer linked by OH groups. A compound of this kind $K_8Ru_2O(NO_2)_{12}$ is mentioned by PASCAL[6]. It does not seem to be identical with the one isolated by us, since it is not orange but yellow, and is supposed to be formed with an excess of nitrite. Like the compound observed by us, it is destroyed by boiling with nitrite.

SUMMARY

The reaction between alkali nitrites and ruthenium salts was studied by means of paper electrophoresis. Conditions for obtaining a single electrophoretic species are described.

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ADSORPTION CHROMATOGRAPHY ON CELLULOSE

VII. THE ADSORPTION ON CELLULOSE OF CHLOROAURIC AND BROMOAURIC ACIDS FROM AQUEOUS SOLUTIONS OF ACIDS

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INTRODUCTION

In two previous papers of this series[1] we have shown that chloroauric and bromoauric acid are adsorbed on cellulose paper and that this kind of adsorption is similar to the adsorption of these compounds on other organic surfaces[2] or to solvent extraction.

The presence of HCl (or HBr) or of their salts leads to the typical salting-out effects usually encountered in solvent extraction. Recently another effect has been studied extensively in solvent extraction. This effect, usually called the "synergic effect", is the alteration of the partition coefficient of the substance to be extracted, on addition of a second solvent or complexant. IRVING AND EDGINGTON[3] have recently discussed such synergism for the extraction of U(VI) with tributyl phosphate and TTA (thenoyltrifluoroacetone) and could show that compound formation with both complexants occurs.

In this paper the effect of the presence of acids other than HCl (or HBr) on the adsorption of chloroauric acid on cellulose will be discussed. We considered it of interest to examine whether synergic effects could be observed in the adsorption of chloroauric acid on cellulose, because the extracted species is a well-known and very stable anion and because, owing to its high solubility in organic solvents, only poor results could be expected from solvent extraction experiments.

EXPERIMENTAL

Chloroauric acid was prepared by dissolving gold(III) chloride in 2 *N* HCl and bromoauric acid by heating a gold(III) chloride solution with conc. HBr on the water bath and subsequently diluting to about *N* HBr.

Whatman No. 3 MM paper was used throughout with ascending development. In our previous work we noted[1] that in high concentrations of acids (HCl or HBr higher than 6 *N* and 3 *N* respectively) there is an increase of adsorption due to the change in the polarity of the solvent. On the other hand there are hydrolysis comets when chloroauric acid is developed in neutral solutions or at acidities below pH 1.

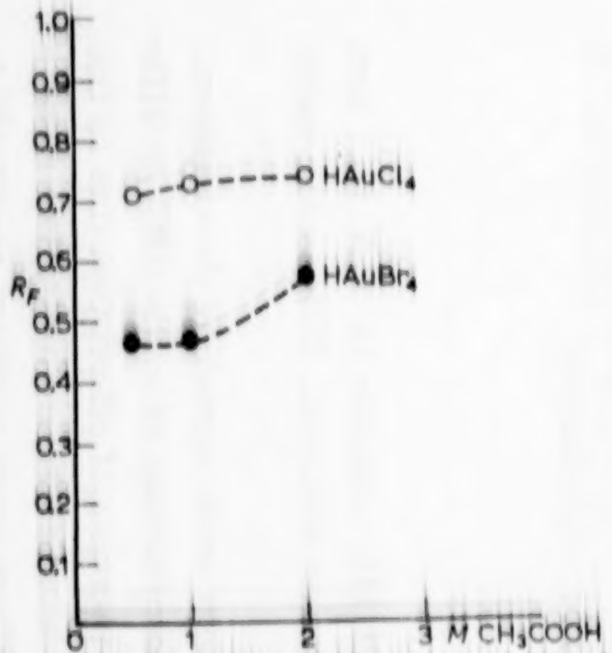

Fig. 1. The R_F values of $HAuCl_4$ and $HAuBr_4$ developed with acetic acid.

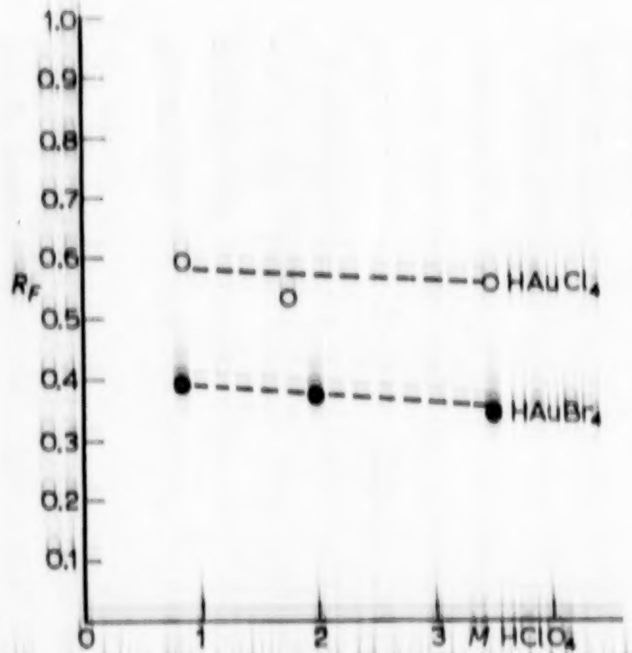

Fig. 2. The R_F values of $HAuCl_4$ and $HAuBr_4$ developed with $HClO_4$.

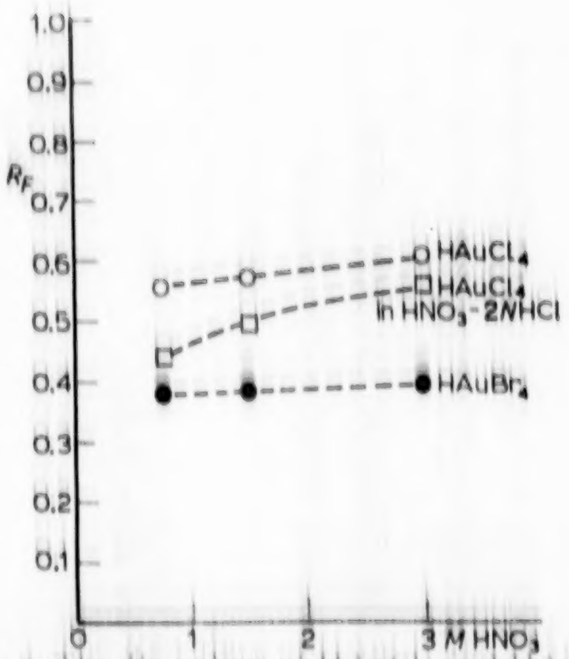

Fig. 3. The R_F values of $HAuCl_4$ and $HAuBr_4$ developed with HNO_3 and a mixture of HNO_3–HCl (2 *N* HCl throughout).

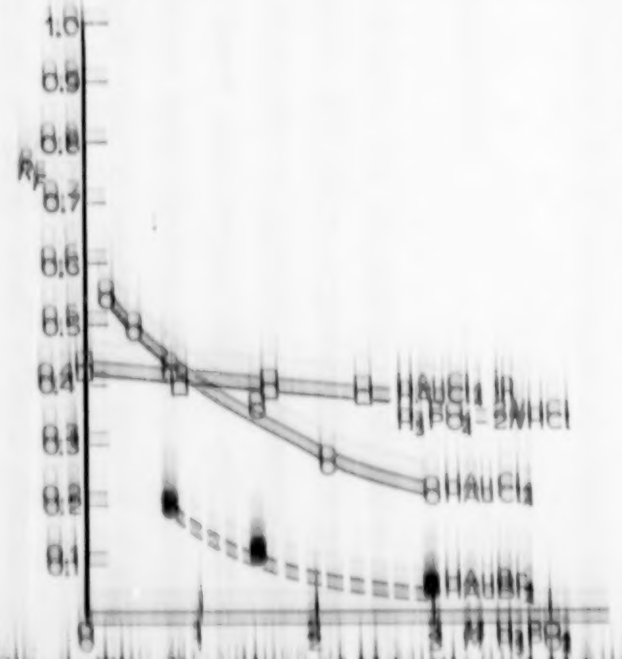

Fig. 4. The R_F values of $HAuCl_4$ and $HAuBr_4$ developed with H_3PO_4 and with a mixture of H_3PO_4–HCl (2 *N* HCl throughout).

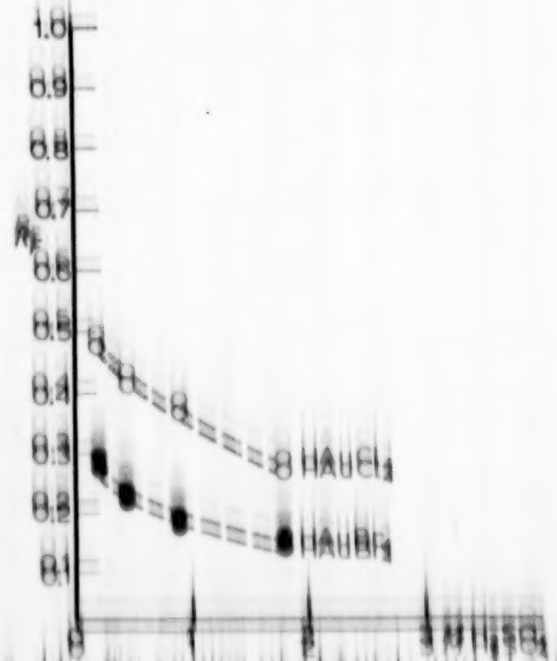

Fig. 5. The R_F values of $HAuCl_4$ and $HAuBr_4$ developed with H_2SO_4.

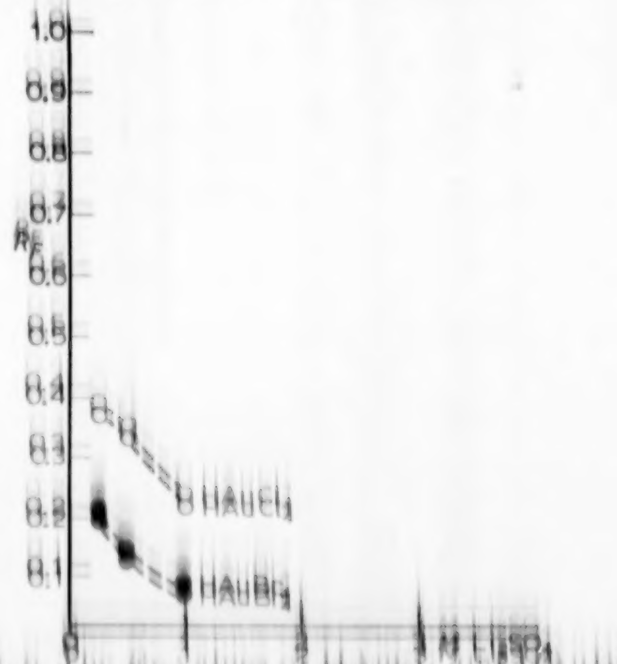

Fig. 6. The R_F values of $HAuCl_4$ and $HAuBr_4$ developed with Li_2SO_4 (slightly acidified with H_2SO_4): 1 *M* Li_2SO_4, 0.36 *N* H_2SO_4; 0.5 *M* Li_2SO_4, 0.18 *N* H_2SO_4 and 0.25 *M* Li_2SO_4, 0.09 *N* H_2SO_4.

Hence we limited our investigations to the range of about 0.5 N to 5 N acid solutions in most cases.

(ii) *The effect of organic acids*

The presence of acetic acid increases the R_F values of (i.e. desorbs) both chloroauric and bromoauric acid as shown in Fig. 1. This effect is similar to that of small amounts of butanol (see ref.[1]) and is probably due to the nonpolar nature of acetic acid. Oxalic acid in presence of HCl (2 N HCl saturated with oxalic acid) increases the R_F value only slightly (from 0.42 to 0.46).

(iii) *The effect of $HClO_4$ and HNO_3*

Both perchloric and nitric acid desorb $HAuCl_4$ and $HAuBr_4$ slightly as shown in Figs. 2 and 3. There is no definite increase in the desorption with an increase of the concentration of HNO_3 or $HClO_4$. In both solvents the colour of the two gold compounds remains unchanged.

(iv) *The effect of H_2SO_4 and H_3PO_4 and sulphates*

There is a marked increase in adsorption when $HAuCl_4$ and $HAuBr_4$ are developed with H_2SO_4, H_3PO_4 or with lithium sulphate as shown in Figs. 4, 5 and 6. As in the interpretation of synergic effects in solvent extraction we can plot the logarithm of the equilibrium constant against the logarithm of the concentration, since R_M (= log (1/R_F − 1)) is directly proportional to the logarithm of the equilibrium constant.

Figs. 7 and 8 show that there is a straight line relationship for the adsorption from H_2SO_4 solutions with a slope of approximately 1/2, a slope of 1 for higher concentrations of H_3PO_4 and a slope of 1/2 for lower concentrations of H_3PO_4 for $HAuCl_4$.

(v) *Adsorption from salt solutions*

Only sulphates were examined as the buffer action of phosphates would complicate the question unduly.

Fig. 9 shows the R_M–log concentration relationship for the adsorption from lithium sulphate solutions that were acidified slightly with H_2SO_4 to prevent hydrolysis of the gold compounds. The slope is 1. There is also an influence due to the cation of the salt: molar Li, NH_4 and Mg sulphates (containing 0.50 N H_2SO_4) gave the following R_F values for $HAuCl_4$ (respectively): 0.22, 0.20 and 0.30.

(vi) *The influence of HCl on the adsorption of $HAuCl_4$ from solutions of other acids*

There is a definite competition between HCl and other acids. H_3PO_4 exerts practically no influence on the R_F value of $HAuCl_4$ in presence of 2 N HCl (Fig. 4). In the case of H_2SO_4 there remains an influence on the R_F value which decreases with the increasing concentration of HCl as shown in Figs. 10 and 11.

Fig. 7 also shows the R_M–log concentration relationship for the adsorption of $HAuCl_4$ from sulphuric acid containing N HCl. There is no change in the adsorption

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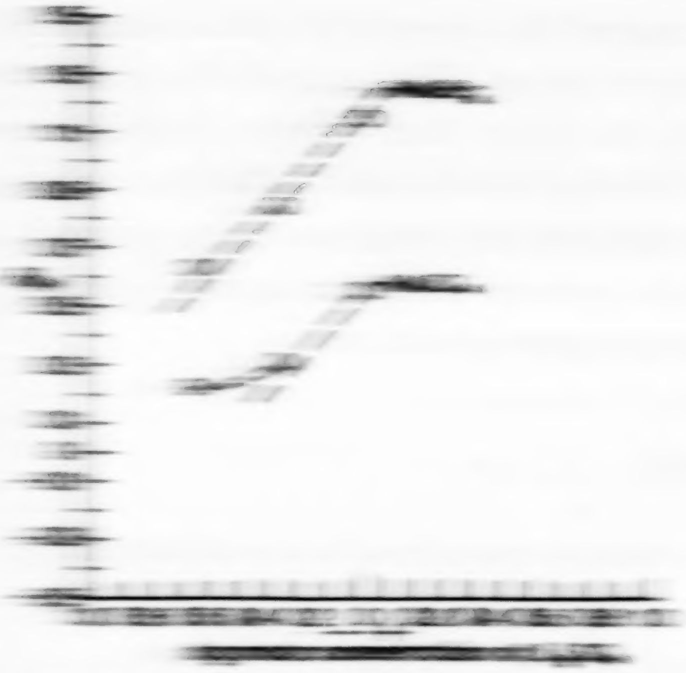

Fig. 9. The R_F values [illegible] H_3PO_4 [illegible]

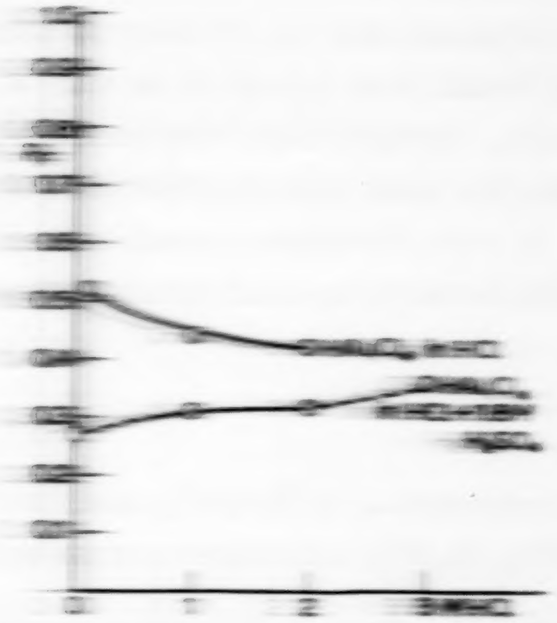

Fig. 10. The R_F values of $HAuCl_4$ in HCl and a mixture of HCl–H_2SO_4, the latter being 1.5 M throughout.

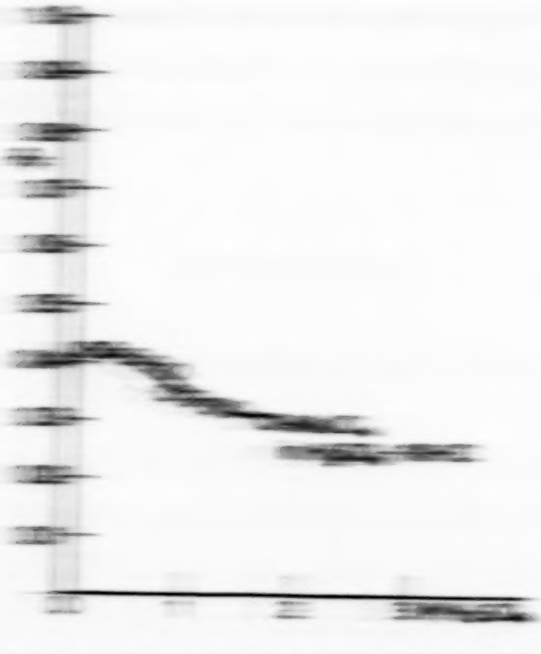

Fig. 11. The R_F values of $HAuCl_4$ in mixtures of H_2SO_4–HCl, the latter being 1 N throughout.

until the sulphuric acid concentration reaches about 1.5 N and then the slope is 1:2. HNO_3 in presence of 1 N HCl still lowers the adsorption of $HAuCl_4$ (Fig. 3).

DISCUSSION

By analogy with the arguments used in interpreting solvent extraction data, it can be assumed that compound formation takes place between $HAuCl_4$ or $HAuBr_4$ and mineral acids such as H_3PO_4 or H_2SO_4 and their salts. The effect seems to be quite general; preliminary results with H_2SeO_4 and metaphosphoric acid also showed a lowering of the R_F values.

So far we cannot offer any evidence for the structure of these compounds. However, the well-known tendency of $HAuCl_4$ to hydrolyse to $HAuCl_3OH$ and to form complexes of the type $HAuCl_3X$ would suggest that phosphoric acid [illegible] and replaces one of the four coordinated chlorogroups. The comparison with HCl would also speak in favour of this. On the other hand neither $HAuBr_4$ nor $HAuCl_4$ change their characteristic colours during development with these various acids. We hope that further work with other methods will elucidate these questions.

It was, however, possible to demonstrate synergic effects or antagonistic effects between several purely anionic species in aqueous solution.

SUMMARY

The adsorption of $HAuCl_4$ and $HAuBr_4$ on cellulose was found to be influenced by H_2SO_4, H_3PO_4 and other compounds.

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THE USE OF TRI-n-OCTYLAMINE-CELLULOSE IN CHEMICAL SEPARATIONS

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(Received January [illegible], [illegible])

In two recent papers[illegible] the potentialities of chromatographic procedures using cellulose paper treated with a liquid anion exchanger such as tri-n-octylamine were discussed. Paper treated in such a way behaves like a film of [illegible] which has a different affinity for the different [illegible] complexes of the elements. Since cellulose is the basic constituent of the paper, our purpose was to investigate the properties of treated cellulose powder beds in order to separate appreciable quantities of substances. Like in the case of paper strips, the cellulose powder was treated with tri-n-octylamine.

EXPERIMENTAL

Preparation of the cellulose powder

Twenty grams of Whatman No. [illegible] cellulose powder were dried for [illegible] hours at [illegible] and then allowed to cool at room temperature in a desiccator. [illegible] of a [illegible] solution of tri-n-octylamine (TNOA) in benzene were shaken for [illegible] with [illegible] the volume of the solution selected for equilibrating the column. The organic phase was then separated and passed through cotton lint to remove any residual water droplets. The dried and cooled cellulose powder was put into a [illegible] bottle and after the addition of the amine solution, the mixture was stirred overnight by means of a magnetic stirrer. After the treatment, the cellulose powder was allowed to settle and then filtered through a [illegible] paper filter. The liquid was allowed to drip off and the wet powder was gently pressed with a paper disk to improve the removal of liquid. Finally the powder was placed between two filter paper sheets in order to completely blot the organic solution that still adhered to the external surface of the powder grains. Afterwards the powder was dried for [illegible] at [illegible] to volatilize the benzene [illegible] and then it was allowed to cool in a desiccator. Before use, the treated cellulose was [illegible] and then put into a column up to the required height; the [illegible] were compressed by carefully pressing the bed with a glass rod.

The columns, which were fitted at the bottom with a sintered glass disk, [illegible] the powder bed [illegible] cross-section area of [illegible].

[illegible]

A number of chemical systems [illegible] amine-cellulose (TOAC) powder [illegible] section. The experimental [illegible]

Separation of Fe^{3+}–Co^{2+}–Ni^{2+}

The adsorption curves of anionic [illegible] nickel does not form anionic [illegible] are highly complexed (D_v about [illegible]) complexed in 3 *M* HCl (D_v about [illegible] D_v < 1). [illegible] chromatography with tri-*n*-octylamine [illegible] had shown how these elements [illegible] 0, 0.5 and 0.97 for iron, cobalt and nickel [illegible] applied for the separation of [illegible] column experiments with cellulose powder.

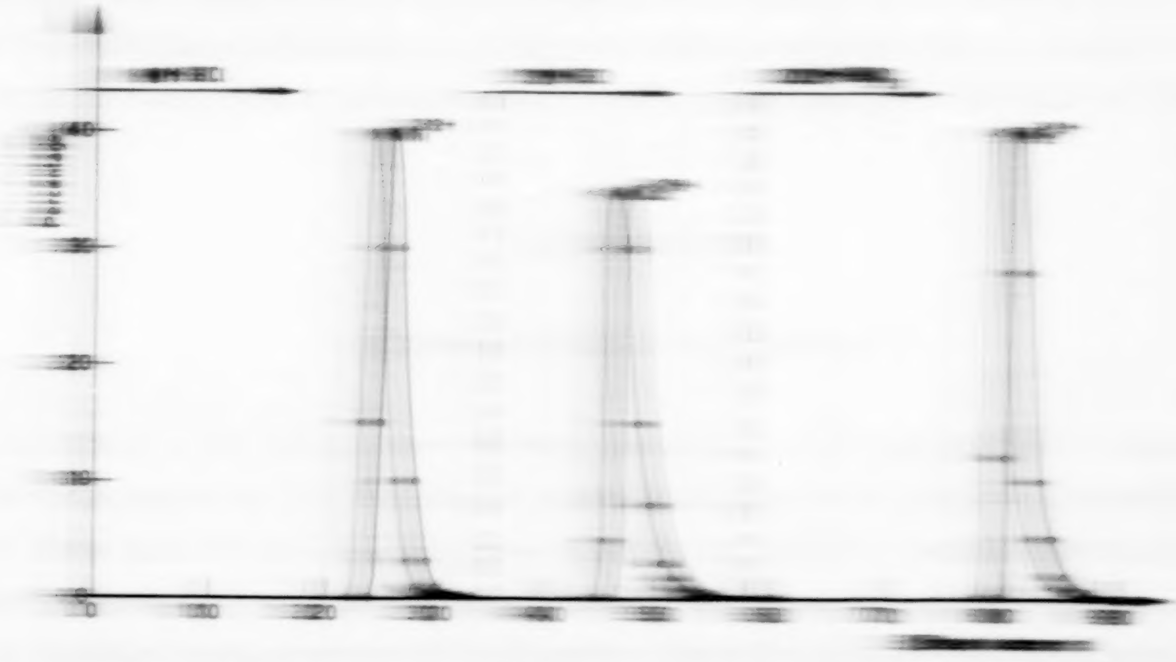

Fig. 1. Separation of Ni–Co–Fe [illegible] effluent *vs.* effluent volume [illegible]

The TNOA solution used [illegible] was pre-equilibrated with 8 *M* HCl [illegible] TNOA in the column itself. The cellulose [illegible] 8 *M* HCl) contained 5 mg of each element. [illegible] adsorbed by the bed, elution with 8 *M* HCl [illegible] yellow immobile band at the top [illegible] downwards very slowly, and nickel [illegible] After nickel had been completely [illegible] finally iron was removed from the [illegible] determined by spectrophotometry. [illegible] Fig. 1.

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Separation of Th–Zr–U^{6+} in a hydrochloric acid medium

The separation of Th, Zr and U^{6+} in a hydrochloric acid medium is possible on the basis of the following considerations[13]: thorium is not complexed by HCl of whatever molarity, zirconium is not complexed by 6 *M* HCl ($D_v < 1$), but is complexed by 10 *M* HCl (D_v about 100), and uranium(VI) is complexed by 6 *M* as well as 10 *M* HCl (D_v about 1000). For these reasons 10 *M* HCl was used to pre-equilibrate the TNOA and the TOA in the column. The bed height was 73 mm and the feed solution (5 ml of 10 *M* HCl) contained Zr (25 mg) + U (35 mg) + Th (1 mg). Thorium was marked with ^{234}Th. During the elution with 10 *M* HCl to remove thorium, uranium gave a yellow band situated at the top portion of the bed. Zirconium was eluted with 6 *M* HCl and finally uranium with 0.5 *M* HNO$_3$. The elution rate was 0.25 ml/min. In the eluted fractions thorium was determined by beta counting, zirconium by complexometry and uranium by spectrophotometry. The elution curve is shown in Fig. 2.

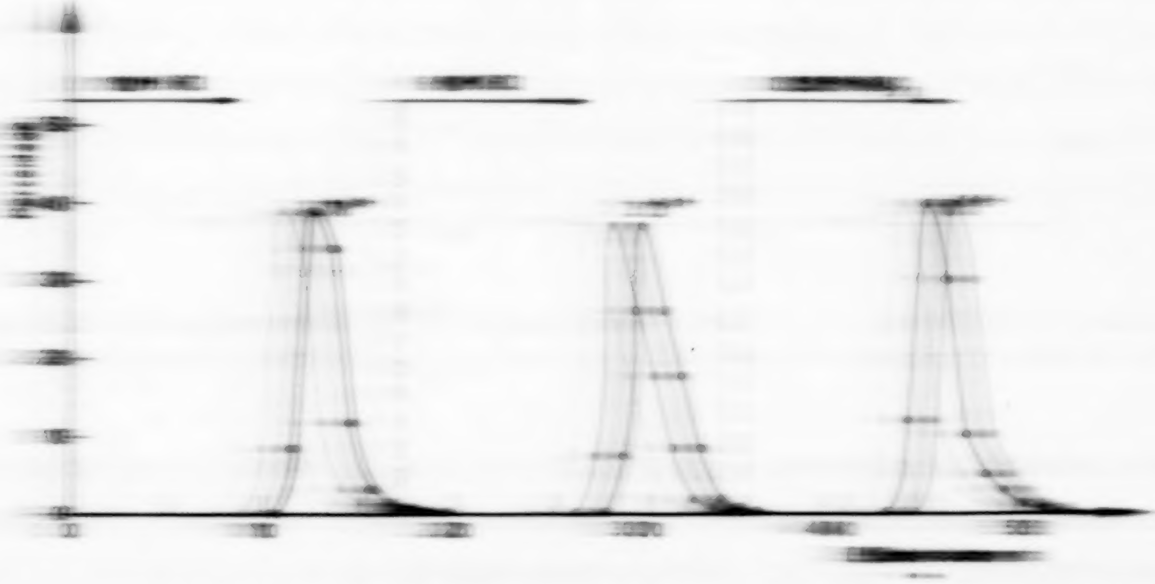

Fig. 2. Separation of Th (1 mg + ^{234}Th)–Zr (25 mg)–U (35 mg). Percentage of initial amount detected in the effluent vs. effluent volume. Bed 73 mm. Flow rate 0.25 ml/min cm^2.

Separation of Zr–U^{6+}–Th with NH$_4$NO$_3$

The separation of these three elements can also be obtained in a medium other than HCl. Sometimes it is necessary to separate small quantities of uranium and thorium from a large amount of zirconium. This is the case, for instance, when trace amounts of the former two elements have to be determined in zirconium ores such as zircon, for geochemical or geochronological purposes. It is convenient therefore to use a procedure by which zirconium is not retained on the bed, while the two trace elements are retained. It has been reported[13,14] that 10 *M* NH$_4$NO$_3$ forms complexes with uranium ($D_v > 100$) and with thorium ($D_v > 100$), but not with zirconium. Furthermore, with 5 *M* NH$_4$NO$_3$ thorium ($D_v > 100$) is more complexed than uranium ($D_v > 10$), so that a separation of these elements can be achieved. A 10 *M* solution of NH$_4$NO$_3$ was used to equilibrate TNOA and TOA.

After the feed solution has been deposited on the top of the bed, washing with a 10 *M* NH$_4$NO$_3$ solution must be carried out to remove zirconium. The overall proceare slightly different according to whether small or large amounts of zirconium are present.

The two cases are described below.

(a) Small amounts of zirconium. At the top of a 1.5 cm high column, 5 ml of a 10 *M* NH_4NO_3 solution containing U (5 mg), Zr (10 mg) and Th (1 mg marked with ^{234}Th) were dispersed. Elution at 0.5 ml/min with 10 *M* NH_4NO_3 was continued until the removal of zirconium was complete. Uranium was eluted with 6 *M* HNO_3 and thorium with 8 *M* HCl. The elution plot is given in Fig. 3.

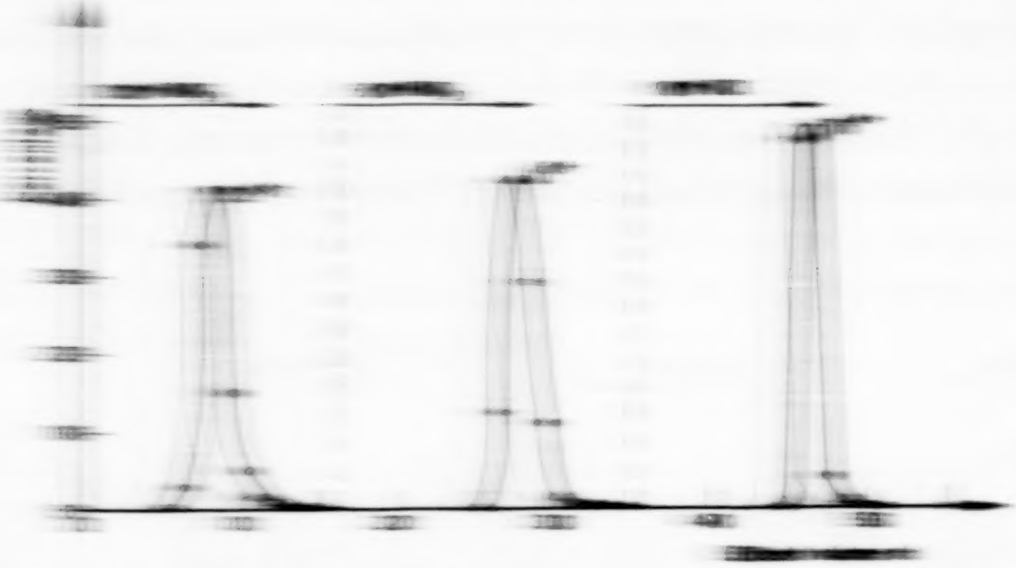

Fig. 3. Separation of Zr (10 mg)—U (5 mg)—Th (1 mg)—^{234}Th. Percentage of initial amount detected in the effluent vs. effluent volume. Bed: 1.5 cm × 1 cm²; elution rate 0.5 ml/min·cm².

(b) Large amounts of zirconium. To simulate the case in which traces of uranium and thorium must be determined in a zirconium ore, such as zircon, the following solution was prepared: Zr (500 mg, which corresponds to about 1 g zircon), U (2 mg) and Th—^{234}Th (2 mg). Because of the relatively high content of zirconium, the feed solution could not be prepared in pure 10 *M* NH_4NO_3. It was possible, however, to obtain a solution by using 2 *M* HNO_3 saturated with NH_4NO_3. This solution (10 ml) was fed to a 5 cm high bed and prolonged washing was carried out with 100 ml of 10 *M* NH_4NO_3. The washing with this solution is necessary to remove almost all the zirconium without contamination with uranium. After that, uranium was eluted with 50 ml of 6 *M* HNO_3 and then thorium with 8 *M* HCl. The recovery of zirconium was about 96%, that of uranium 99% or higher and that of thorium was 100%.

Separation of U^{6+} and Th from Ti^{4+} and Fe^{3+}

Another interesting case in the chemistry of some titanium ores is the determination of traces of uranium and thorium in ilmenite. Separation of U^{6+} and Th from the main constituents of the ore (Ti and Fe) can be achieved by following the procedure described for the separation of large amounts of zirconium from uranium and thorium. In fact neither titanium nor iron give nitrate complexes and therefore they are not retained by TOAC.

Separation of La–U–Th

It has been reported[24] that lanthanum is complexed to a certain extent by NH_4NO_3. Nevertheless, since uranium and thorium are more strongly complexed in 10 *M* NH_4NO_3 and firmly retained by TOAC, lanthanum can be eluted first by prolonged washing with 10 *M* NH_4NO_3. The procedure is the same as for the separation of Zr–U–Th.

Separation of Zr–La

As mentioned above, lanthanum is slightly complexed by 10 *M* NH_4NO_3, and this can be exploited to separate this element from zirconium which is not complexed at all. To test the operating conditions, a feed solution was prepared containing La (5 mg) and Zr — ^{95}Zr (2 mg) in 5 ml of 10 *M* NH_4NO_3. The feed solution was applied to the top of a 13 cm high bed and elution was started with 10 *M* NH_4NO_3 at 0.2 ml/min. After about one column volume, zirconium began to be collected in the eluted liquid and, immediately after zirconium had been completely eliminated, lanthanum appeared and was present for about three column volumes. While zirconium was determined by beta counting of ^{95}Zr, lanthanum was evaluated by complexometry. As shown in Fig. 4, zirconium presents a narrow peak, and lanthanum a flat elution curve. To speed up the removal of lanthanum, as soon as the collection of zirconium is completed the elution can be carried out with HNO_3 or HCl.

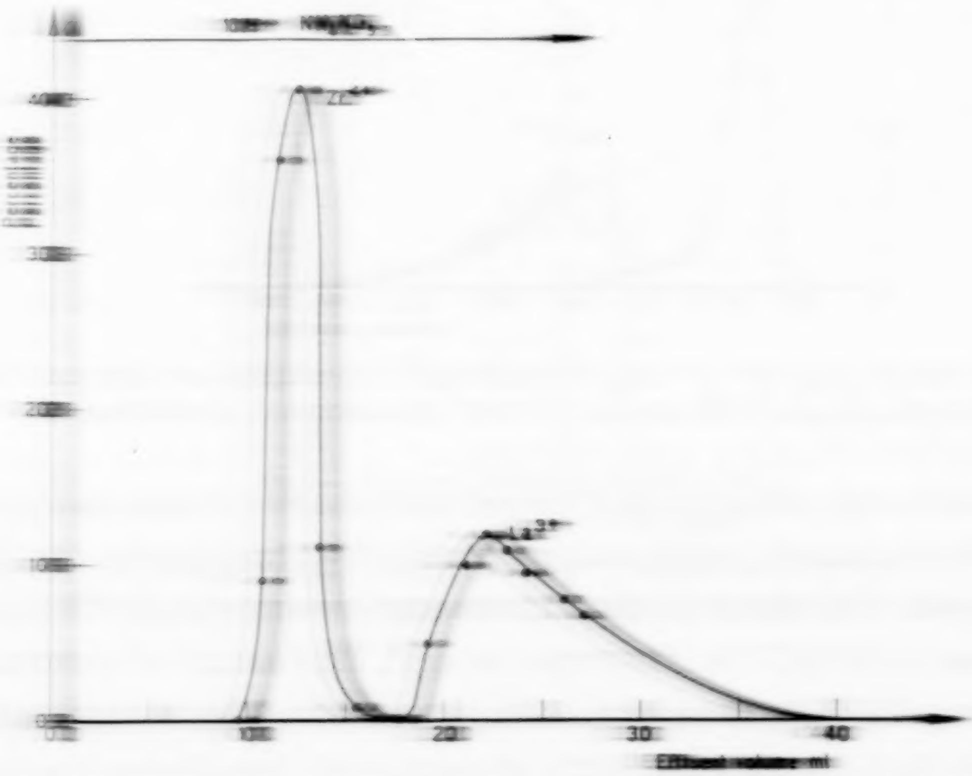

Fig. 4. Separation of Zr (2 mg) — ^{95}Zr–La (5 mg). Percentage of initial amount detected in the effluent vs. effluent volume. Bed: 13 cm × 1 cm²; elution rate 0.2 ml/min.cm².

The conditions reported in the last two examples can be successfully applied to the separation of the four elements Zr, La, U and Th, namely by using sequential elution with 10 *M* NH_4NO_3, 6 *M* HNO_3, and 8 *M* HCl.

Separation of Zr–Hf

In spite of their chemical similarity, zirconium and hafnium have been easily separated from their mixtures by means of solvent extraction with TNOA in a hydrochloric–nitrate medium[5]. The extraction coefficient E_a° is larger than 2 for zirconium and smaller than 0.2 for hafnium. The same hydrochloric–nitrate medium was also used in the paper chromatography with a TNOA-treated paper[1], the R_F values being 0.25 for zirconium and 0.88 for hafnium. From the present experiment it appears that a good separation can be obtained also by using a TOAC column. In this case the TNOA solution used for the treatment of the cellulose powder, as well as the TOAC powder placed in the column, were pre-equilibrated with 8 M HCl mixed with 5% (vol.) of conc. HNO_3. The feed solution contained Zr (10 mg) and 3% (mol.) of Hf marked with ^{181}Hf. After the feed solution had been adsorbed by the column (bed height 30 cm), elution with 8 M HCl + 5% conc. HNO_3 was started at 0.2 ml/min. As the elution curve (Fig. 5) shows, hafnium, which was determined by beta counting, appeared in the effluent after about one column volume and was removed very rapidly, while zirconium, which was determined by complexometry, presented a less pronounced peak. The shape of the elution curve is very similar to that obtained with Dowex 2 and 9 M HCl by HUFFMAN *et al.*[6]. The elution of zirconium can be accelerated by using HNO_3 as the eluent, after hafnium has been completely removed.

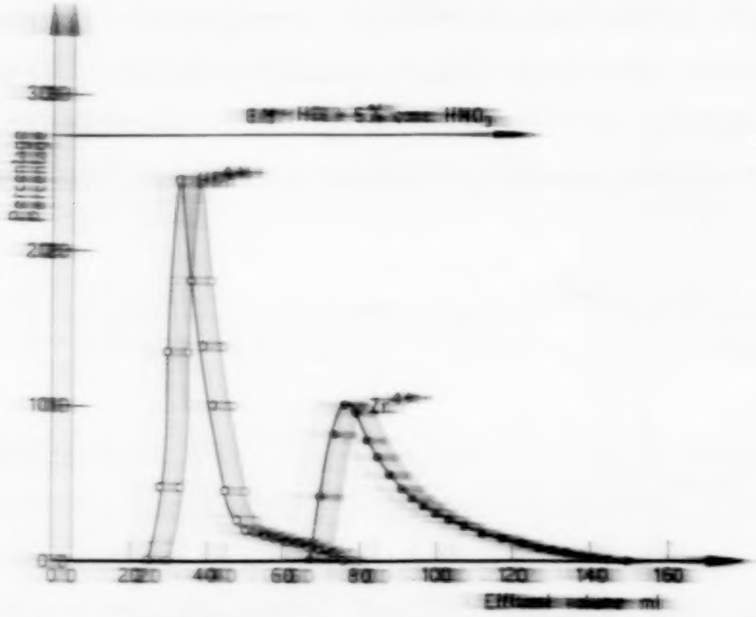

Fig. 5. Separation of Hf (0.3 mg)–Zr (9.7 mg). Percentage of initial amount detected in the effluent vs. effluent volume. Bed: 30 cm × 1 cm²; elution rate 0.2 ml/min.cm².

Evaluation of the exchange capacity of TOAC

By treating the cellulose powder with TNOA solutions of different concentrations, some samples of TOAC with different exchange capacities were obtained. The exchange capacity was determined by allowing a 0.1 M HCl solution to flow through a 12.5 cm high bed (1 cm² cross-section), at 0.3 to 0.4 ml/min. The number of equivalents of HCl retained by the bed was estimated by determining the acidity of the effluent by titration with 0.1 M NaOH.

The results are collected in Table I, where the exchange capacity, in mequiv. per ml wet TOAC, are reported in comparison with the molarity of TNOA (0.05 to 1 M) used in the treatment of the cellulose powder. The TOAC prepared with TNOA up to 0.5 M revealed a very good stability with 0.1 M HCl, and no amine was removed from the column by the acid. The TOAC prepared with 1 M TNOA showed an excess of amine and a certain amount of this was removed by the acid.

TABLE I

EXCHANGE CAPACITY OF TOAC AS A FUNCTION OF THE MOLARITY OF TNOA IN THE SOLUTION USED FOR TREATMENT OF THE POWDER

Bed cross-section 1 cm², height 12.5 cm, flow rate 0.4 ml/min.

Exchange capacity mequiv./ml wet material	TNOA molarity (treatment solution)
0.030	0.05
0.045	0.10
0.130	0.25
0.210	0.50
0.250*	1.00

* Determined by saturation with $(UO_2Cl_4)^{2-}$.

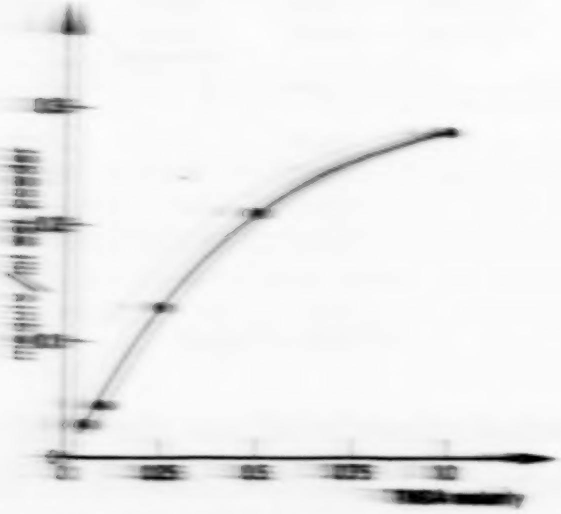

Fig. 1. Capacity of the cellulose bed (mequiv./ml wet powder) as a function of the molarity of the TNOA solution used in the treatment of the cellulose powder.

To check the values of the exchange capacity, the TOAC treated with 0.5 M TNOA was also investigated by saturating the bed with an 8 M HCl solution containing uranium. In view of the fact that the complex $(TNOA)_2UO_2Cl_4$ is formed, the result is identical to that obtained with 0.1 M HCl. The exchange capacity of the TOAC treated with 1 M TNOA was only determined with the uranium solution. In both these latter cases the bed was pre-equilibrated with 20 ml of 8 M HCl and then treated with a solution containing 12 mg/ml of uranium in 8 M HCl. The flow rate of the solutions was kept at 0.4 ml/min. The uranium capacity of the wet TOAC is 24 mg U/ml and 33.6 mg U/ml respectively for the 0.5 M TNOA type and for the 1 M type.

Because the common anionic resins exhibit an exchange capacity of about 1 mequiv./ml (wet resin), the results obtained with saturated TOAC are approximately 3 or 4 times lower.

For purposes of comparison with the aminated cellulose powder, Whatman diethylaminoethyl-cellulose (DEAE) was selected. DEAE is a commercially available aminated cellulose, widely used for the chromatographic separation of amino acids, proteins, enzymes, nucleic acids and hormones[7,8]. The exchange capacity, according to the suppliers, is 0.3 mequiv./ml, but this material did not prove very suitable for the retention of uranium. In fact, when DEAE was pre-equilibrated with uranium in 6 N HCl, a brown zone formed in the bed which travelled rapidly with the front. The effluent was contaminated by organic substances and the uranium capacity of the wet DEAE proved to be 20.8 mg U/ml (about 0.17 mequiv./ml). Furthermore, uranium is much less strongly retained by DEAE than by TOAC. In fact, 6 N HCl completely removed uranium from DEAE by elution with less than two column volumes, while with TOAC the release was slight and very slow. The results are summarized in Table II.

TABLE II

COMPARISON OF TOAC WITH DEAE AS REGARDS RETENTION OF URANIUM FROM HYDROCHLORIC SOLUTIONS

	Theoretical mequiv./ml	*U (mg) mg/ml*	*Exchange capacity (exp.) mequiv./ml*	*U release with 6 N HCl*
TOAC	—	33.6	0.28	very slow
DEAE	0.30	20.8	0.17	fast

CONCLUSIONS

From the experiments reported above it follows that good separations of many elements can be achieved by using TOAC columns instead of the common anionic resins.

Though TOAC has an exchange capacity that is three or four times lower than that of the common anionic resins, some advantages of TOAC can be pointed out. The main advantages are:

(1) Owing to the white colour of the TOAC powder, the position and the movement of coloured zones can be followed in the case of elements that form coloured bands in the bed.

(2) Since the active functional groups are concentrated at the surface of the powder grains, and the grain size is very small, TOAC has a high ability to fix also large molecules, which often cannot penetrate the complex structure of anionic resins.

(3) TOAC generally gives very narrow elution peaks and exhibits an exchange rate higher than that achieved with resins. Furthermore, TOAC can be successfully used in inorganic systems where elements that form anionic complexes must be sep-

rated in inorganic acids of high concentration. In fact, unlike DEAE, TOAC is very stable also in HCl 8 to 10 *M* and in HNO_3 5 to 7 *M*.

When required, TOAC can be easily regenerated and transformed into different forms with various acids.

(4) When working with radioactive traces, a further advantage of TOAC powder is that it can easily be burned and the residue can be conveniently mounted as a source for radioactive counting. Obviously, this advantage is common to all the cellulose products, whereas the resins are less easily burned.

SUMMARY

A method is described for the preparation of tri-n-octylamine–cellulose (TOAC). A column of this substance behaves like an anionic resin column and permits the separation of many chemical elements. The following separations are given as examples: Fe–Co–Ni, U–Th–La, U–Th–Zr, La–Zr and Zr–Hf. The exchange capacity was evaluated and found to be three to four times lower than that of the common anionic resins. Some advantages are also indicated. TOAC was compared with Whatman's diethylaminoethyl-cellulose (DEAE) as regards the retention of uranium from a very acid solution.

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Notes

Anion exchange behaviour of some rare earths in dilute sulphuric acid solutions containing ethanol

Although rare earths are not appreciably adsorbed by strongly basic anion exchangers from hydrochloric[1,2], nitric[3,4] and sulphuric[5,6] acid solutions, adsorption from dilute nitric acid–ethanol and dilute hydrochloric acid–ethanol solutions has been reported[7]. The differences between the distribution coefficients of Y, Nd and La in the nitric acid–ethanol media were sufficient to permit satisfactory separations. These findings prompted the author to investigate the anion exchange behaviour of some rare earths (Y, Nd, Pr and La) in dilute sulphuric acid solutions containing ethanol.

Experimental

Materials. Air-dried Amberlite IRA-400 8 ×, 100–200 mesh in the SO_4 form; Specpure Y, Nd, Pr and La oxides; A.R. H_2SO_4 (s.g. 1.84) and C.P. ethanol (95%).

Distribution coefficient measurements. Adsorbabilities were determined at room temperature (23 to 25°) by the batch equilibrium method using 0.25 g resin as a slurry with 20 ml of the appropriate sulphuric acid–ethanol solutions containing 2 mg of Y, Nd, Pr or La in a stoppered 100 ml erlenmeyer flask for 20 h. The following H_2SO_4–ethanol solutions were investigated:

(a) 80% ethanol containing varying concentrations of H_2SO_4, 0.1, 0.01 and 0.001 N.

(b) 0.01 N H_2SO_4 containing varying concentrations of ethanol (0, 25, 50 and 80%).

From analyses of the solution phase before and after equilibration, distribution coefficients K_D $\left(\frac{\text{amount of rare earth per g resin}}{\text{amount of rare earth per ml solution}}\right)$ were computed. Rare earths were determined spectrophotometrically with bromopyrogallol red[8].

Blank runs showed no significant adsorption by the container walls. All equilibrations were carried out in triplicate and the average of the triplicate results is reported. The average spread of the triplicate determinations was ±1%.

Results and discussion

The distribution coefficients of Y, Nd, Pr and La in 80% ethanol containing various concentrations of H_2SO_4 are shown in Fig. 1.

For a given rare earth, the adsorbability decreases with increasing sulphuric acid concentration. The adsorption of yttrium by a strongly basic anion exchanger from dilute ammonium sulphate solutions was found to decrease with increasing ammonium sulphate concentration[9].

At constant N H_2SO_4 the extractability of the rare earths increases in the order:

$$Nd < Pr < Y < La.$$

With the exception of Y, this sequence is also the order of increasing ionic radii in the rare earth series (Y^{3+} (0.93 Å), Nd^{3+} (1.04 Å), Pr^{3+} (1.06 Å) and La^{3+} (1.14 Å)).

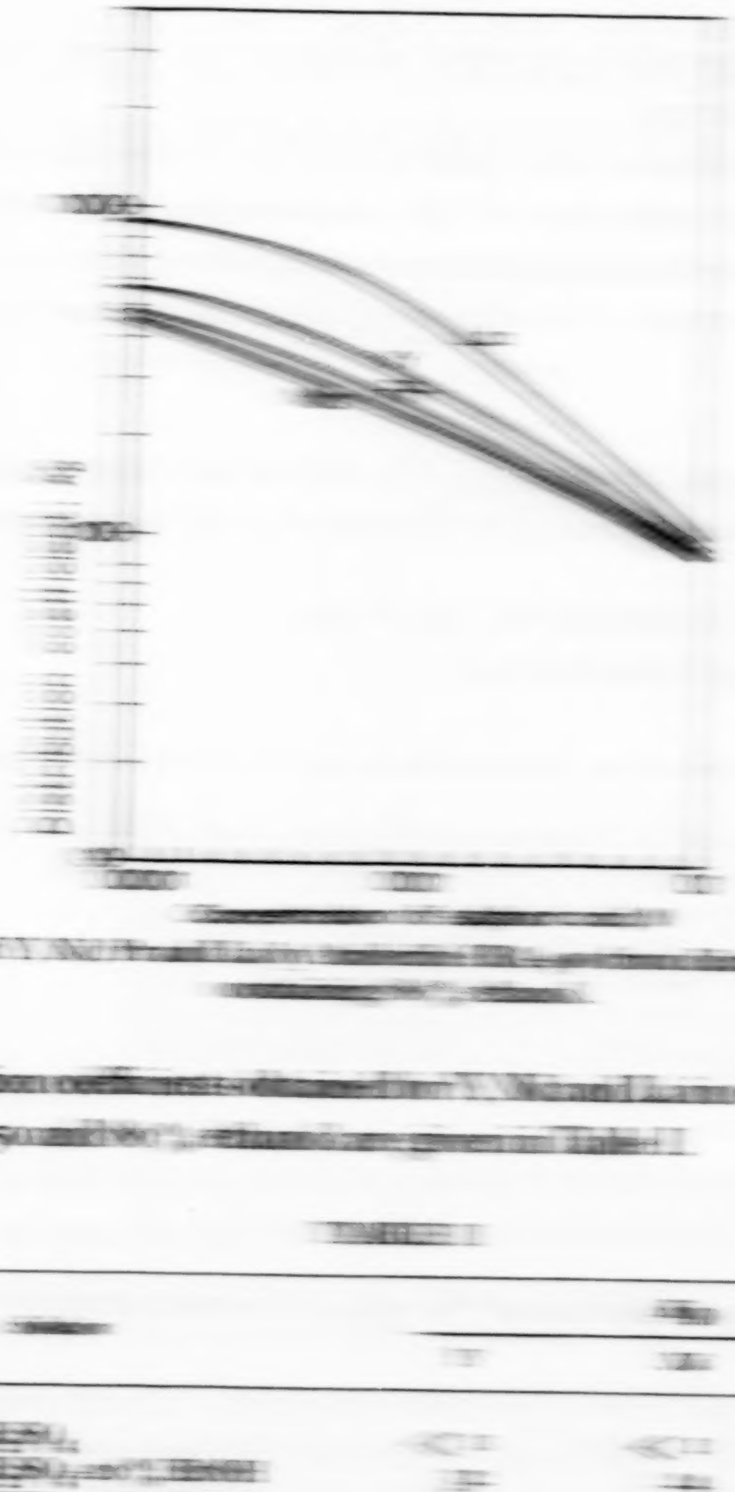

Fig. 1. Adsorption of Y, Nd, Pr and La on Dowex 50W-X8 from sulphuric acid solutions containing 80% ethanol.

The distribution coefficients obtained for Y, Nd and La in 0.01 N H_2SO_4 solutions containing 0, 20, 50 and 80% ethanol are given in Table I.

TABLE I

Eluant	K_D		
	Y	Nd	La
0.01 N H_2SO_4	≪1	≪1	≪1
0.01 N H_2SO_4, 20% ethanol	7	14	34
0.01 N H_2SO_4, 50% ethanol	80	100	170
0.01 N H_2SO_4, 80% ethanol	395	720	495

From Table I it is seen that as the percentage of the organic component of the solution was increased, rare earth elements were adsorbed more strongly by the resin.

For satisfactory column separation, separation factors (ratio of distribution coefficients) as different as possible from unity are desirable. Suitable eluant concentrations to use for some rare earth separations are collected in Table II.

TABLE II

[illegible]	[illegible]	[illegible]
Nd–La	[illegible]	0.1—0.01 N H_2SO_4–50% EtOH
Y–La	[illegible]	0.1—0.01 N H_2SO_4–50% EtOH
Nd–Y	[illegible]	0.01 N H_2SO_4–50% EtOH
Pr–Y	[illegible]	0.01 N H_2SO_4–50% EtOH

Separations would be expected to be slow because of the strong absorption of rare earths by the anion exchanger.

The distribution coefficient data plotted in Fig. 1 offer little prospect for an effective anion exchange separation of the neighbouring rare earths Nd and Pr.

Dilute sulphuric acid-ethanol eluants are unsuitable for the separation of macro amounts of rare earths owing to the limited solubility of rare earth sulphates in these media.

The author wishes to thank Professor [illegible] for helpful discussion and the South African Atomic Energy Board for the award of a Senior Bursary.

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Received May 15th, 1961

J. Chromatog., 6 (1961) 452–454

A fog precipitator for gas chromatography

The flow of hot carrier gas from a gas chromatographic column usually results in the sample emerging as a fog. Flow rates commonly used make efficient collection using conventional cold traps almost impossible, yields often being as low as 60%. The apparatus described below makes possible recoveries of over 98%.

In this laboratory, eluting materials are collected from columns of between 1/4 and 1 in. diameter, with nitrogen flow rates of between [illegible] ml/min and 2 l/min, and temperatures from 20 to 220°.

The apparatus consists of a glass cold trap and a metal precipitator containing an electric field supplied from a source of constant current sufficient to develop a field strength of from 5,000 to 12,000 [illegible].

Fig. 1 shows a cold trap designed to collect from a 1-in. diameter column which often produces 1 g of sample over a period of 5 min, with a nitrogen flow of 1 l/min. Smaller traps can be used for the collection of smaller quantities, but care should be taken to see that the trap size is reduced to scale, because the ratios of the tube diameters are critical if one is to eliminate losses by causing turbulence of the fog in the converging tubes. It has been found convenient to construct a simple jig to enable any number of cold traps to be made, each with two B_{14} sockets spaced the same distance from each other and at the same height, so that they may be exchanged quickly. These methods are necessary since peaks do not emerge very quickly from our columns and the volumes are too small to justify the construction of automatic collection apparatus.

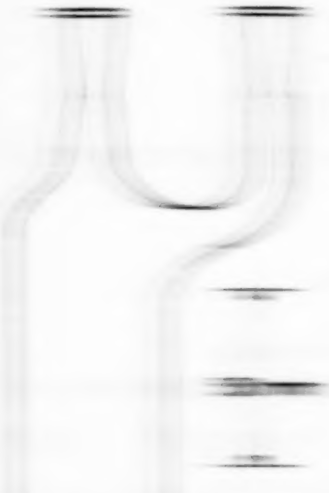

Fig. 1. Cold trap with two standard B_{14} sockets.

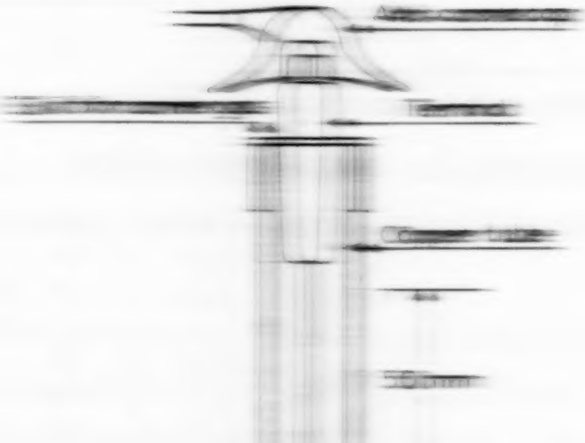

Fig. 2. Metal precipitator. [illegible]

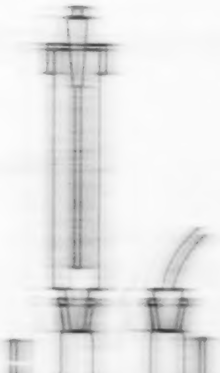

Fig. 3. The assembled apparatus. Ceramic to metal lead through sealed terminal (E.L.G. type CS 115/2). Copper tube 25 mm in diameter.

The metal precipitator (Fig. 2) does not always require cleaning after each peak of eluted material because the geometry of trap and precipitator and the shape and extent of the electric field are such that only a small percentage of unusually high vapour concentrations ever enter the precipitator. Fig. 3 shows the assembled apparatus. It should be noted that gas enters the side arm of the trap.

High voltage for the precipitator is derived from a radio frequency oscillator, the output of which is rectified to direct voltage by a valve (type U25), and taken by a very well insulated cable to the central electrode, as shown in Fig. 4. This method of generating a high voltage offers advantage over mains frequency circuits, since the energy content is less at source and therefore the power available is limited if the output should be presented to a low resistance such as the human body, although of course every effort should be made to avoid receiving a shock.

Current flowing through the precipitator is developed across "R" (Fig. 4) to provide feedback and give a measure of automatic constant current control. The

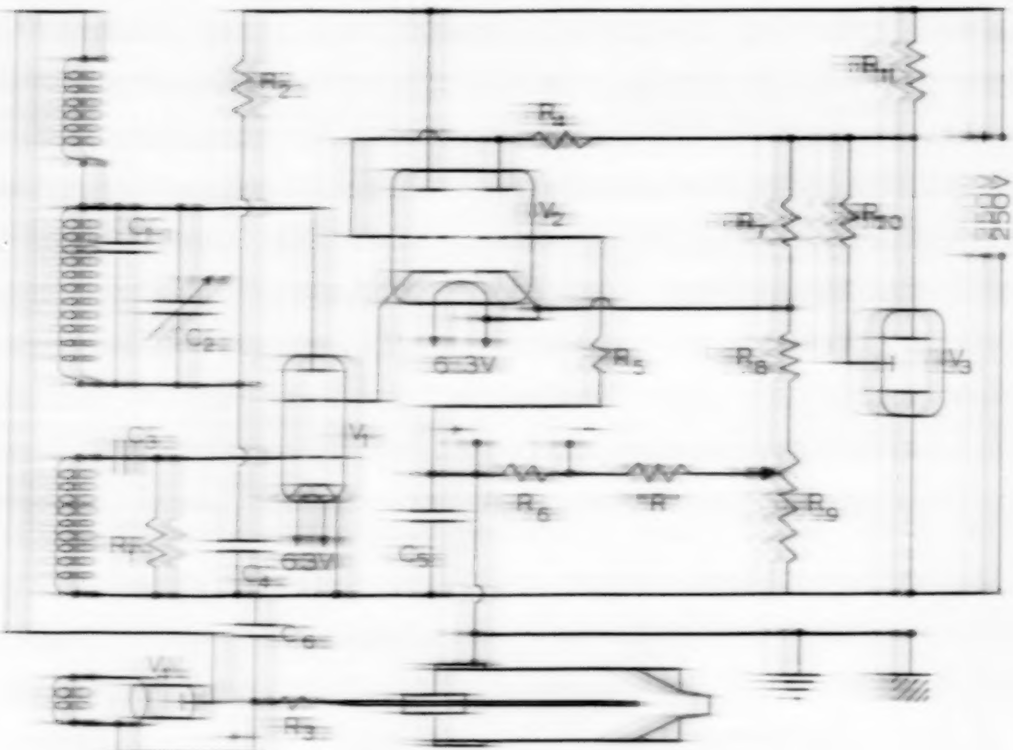

Fig. 1. Circuit diagram. Resistors, all within 10%:

R_1	47	kΩ	1/4 W		R_7	33	kΩ	2 W
R_2	220	Ω	1/2 W		R_8	1.5	kΩ	1/2 W
R_3	470	kΩ	1 W		R_9	5	kΩ	5 W. Wirewound. "Set Current" control.
R_4	680	kΩ	1 W					
R_5	470	kΩ	1/4 W		R_{10}	1	MΩ	1/2 W
R_6	22	kΩ	1/4 W		R_{11}	10	kΩ	5 W
					"R"	220	kΩ	1/4 W

Capacitors, all within 20%:

C_1 0.0002 µF Mica, 1000 V		C_4 0.05 µF 500 V
C_2 0.001 µF Variable, 1000 V. Frequency control.		C_5 0.25 µF 300 V
C_3 0.001 µF 300 V		C_6 0.001 µF 12 kV

Valves:

V_1 6V6 or 6P25 or 6BW6
V_2 6SN7 or B65 or ECC32 or 12AU7 or B329 or ECC82
V_3 150B3 or QS150/15 or CV287
V_4 U25

Coil:

"NERA" R.F. E.H.T. Coil. 8–12 kV.
(Tyer and Co. Ltd., Merrow Siding, near Guildford, Surrey, England.)

range of current available (approx. 10 to 100 µA) is determined by the setting of the "Set Current" control (R_9), a potentiometer mounted on the front panel, and the reference voltage across this component. If required, current may be further increased by decreasing the value of "R". Automatic control is achieved by using one half of a valve (type 6SN7) to amplify changes of voltages across "R" and applying these changes to the other half which is used to adjust the screen grid of the oscillator valve (type 6V6). The high tension supply of 250 direct volts is required to provide a current which varies between 30 and 50 mA during normal operation. Since the reference tube (150B3) cannot correct the oscillator for mains supply variations it is preferable to supply the H.T. from a stable source within the limits of 230 to 300 V, although the author has found a simple unregulated power supply to be adequate.

When setting up the apparatus, the "Set Current" control is adjusted for maximum current and the oscillator frequency control (0.001 μF variable capacitor) is adjusted until a flash-over discharge can be heard from within the precipitator, with the carrier gas flowing. This capacitor is then readjusted until the discharge ceases. The "Set Current" control may now be used.

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Received May 23rd, 1961

J. Chromatog., 6 (1961) 454–457

Comparison of two partitioning phases for gas–liquid chromatography with two types of detectors utilizing four halogenated hydrocarbons

Several halogenated hydrocarbons have been separated by gas–liquid chromatography. Percival[1] separated trichlorofluoromethane and dichlorofluoromethane using di-isooctyl phthalate as the liquid stationary phase and obtained quantitative recoveries of 98–102%. Zweig and Archer[2], Zweig, Archer and Rubenstein[3], and Coulson, Cavanagh, De Vries and Walther[4] utilized silicone grease as the stationary phase for the separation of organo-halogenated compounds.

The present work was undertaken to investigate a suitable column packing for the efficient separation of *cis*-, and *trans*-1,3-dichloropropene, 1,2-dichloropropane, and 1,2-dibromo-3-chloropropane. Data are presented on a comparison of two column packings at two temperature levels together with a comparison of the sensitivities of the hot-wire katharometer detector and the microcoulometer cell.

Experimental

Apparatus and procedure. Gas-chromatographic data were obtained by two types of apparatus: (a) Burrell Kromo-Tog, Model K-1 equipped with a hot-wire katharometer detector and an 8 ft. × 0.25 in. o.d. U-shaped glass column, using helium as the carrier gas. (b) Microcoulometer gas chromatograph Model G-100 (Dohrmann Instruments Company, Palo Alto, California) using a 6 ft. × 0.25 in. o.d. spiral aluminum or stainless steel column and nitrogen as the carrier gas.

Column packings were (a) silicone grease, 20% w/w (Dow-Corning, high methyl polymer) and (b) di-*n*-decyl phthalate, 30% w/w (Eastman Organic Chemicals); each coated on 30–60 mesh acid-washed Chromosorb (Johns-Manville).

Chemical compounds were 1,2-dichloropropane (b.p. 95–97°), *cis*-1,3-dichloropropene (b.p. 104–105°), *trans*-1,3-dichloropropene (b.p. 110–111°), and 1,2-dibromo-3-chloropropane (b.p. 196°).

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The compounds were purified by gas chromatography using a di-n-decyl phthalate column either at 85° or 122°. The desired fractions were collected in acetone–dry ice cold traps and qualitatively identified by infrared analysis. The infrared spectra of the four compounds were similar to published spectra or available reference compounds after chromatographic purification.

Results

Table II shows the data for the relative retention volumes of the halogenated hydrocarbons studied. The data were obtained by means of a gas chromatographic apparatus equipped with a hot-wire detector and an 8-foot column. Table III illustrates the relatively greater sensitivity of the microcoulometer detector when compared to the hot-wire detector which was arbitrarily expressed as unity.

TABLE II

RELATIVE* RETENTION VOLUMES ON HOT-WIRE KATHAROMETER DETECTOR WITH EIGHT-FOOT COLUMN**

Column		Silicone gum		Di-n-decyl phthalate	
Compound	b.p. °C	85° ml	122° ml	85° ml	122° ml
1,2-Dichloropropane	95–97	30.0	13.5	128	40.8
1,3-Dichloropropene (cis-)	104–105	35.3	12.0	100	51.0
1,3-Dichloropropene (trans-)	110–111	40.8	15.0	272	63.4
1,2-Dibromo-3-chloropropane	196	324	39.0	>720	340

* Relative retention volume was calculated by multiplying the carrier gas flow rate, as measured at the column exit, by the total retention time as measured from the point of sample injection to the point of total elution of each component.

** Helium flow rate 60 ml/min.

TABLE III

COMPARISON OF RELATIVE SENSITIVITY OF KATHAROMETER AND MICROCOULOMETER DETECTORS USING A DI-n-DECYL PHTHALATE COLUMN

Compound	Detector				Sensitivity ratio[b]
	Katharometer		Microcoulometer		
	att.[a]	µmoles	att.[a]	µmoles	
1,2-Dichloropropane[c]	32	0.013	20	0.75	58 ×
1,3-Dichloropropene[c] (cis-)	37	0.013	20	0.66	51 ×
1,3-Dichloropropene[c] (trans-)	32	0.013	15	0.72	56 ×
1,2-Dibromo-3-chloropropane[d]	32	0.006	31	0.02	6 ×

[a] 1/8 resistance of the attenuator.

[b] Relative greater sensitivity of microcoulometer detector based upon katharometer detector as unity.

[c] 85°.

[d] 122°.

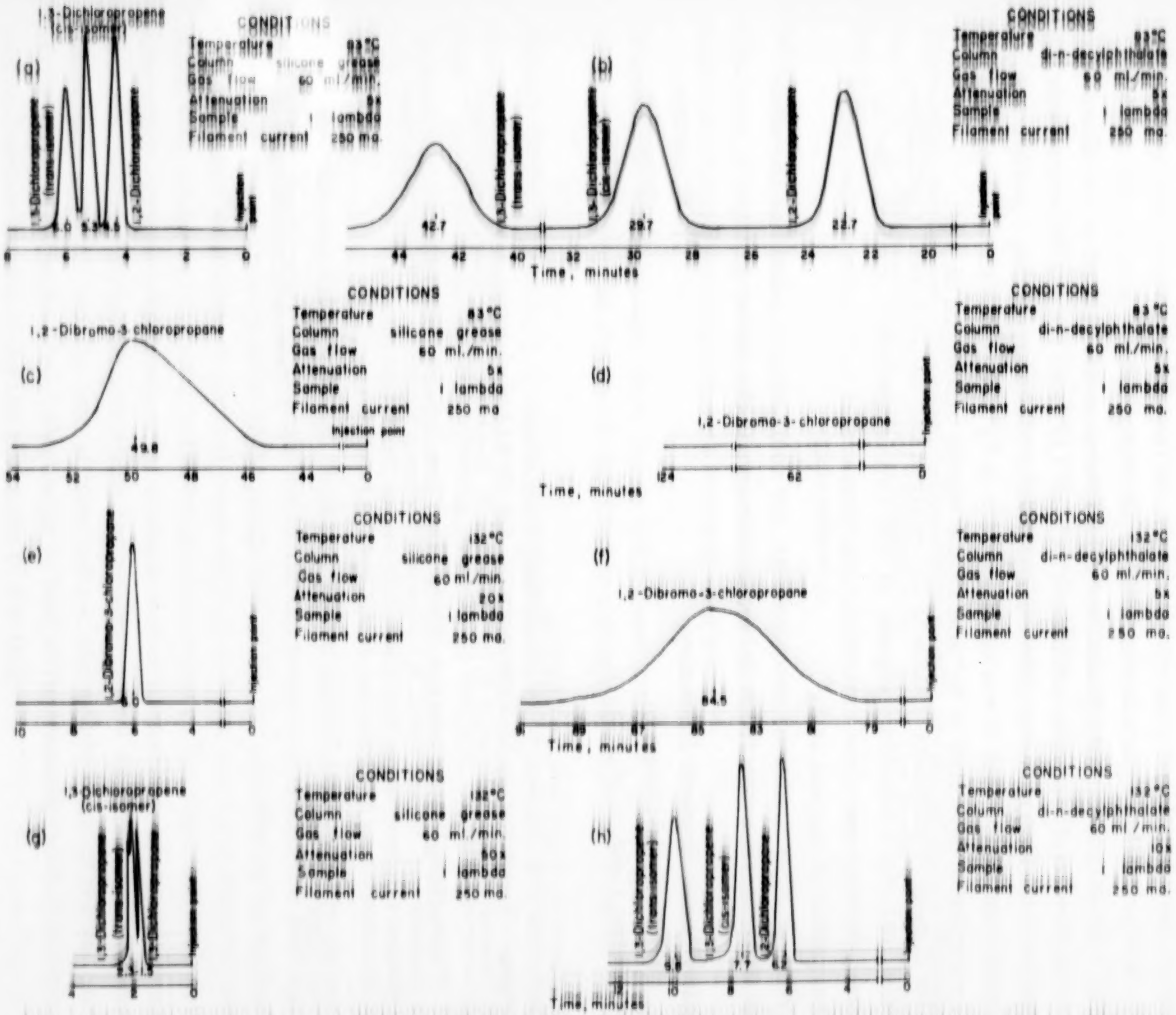

Fig. 1. Chromatograms of *cis*-1,3-dichloropropene, *trans*-1,3-dichloropropene, 1,2-dichloropropane, and 1,2-dibromo-3-chloropropane. Chromatograph: Burrell Model K-1. Columns: Silicone grease (Dow-Corning high methyl polymer) and di-*n*-decyl phthalate (Eastman Organic Chemicals). Column length: 8 ft. Column temperatures: 83° and 132°. Helium flow rate 60 ml/min.

Coulson *et al.*[6,7] have discussed the technique of gas–liquid chromatography in combination with the microcombustion of the chromatographed sample followed by detection with microcoulometry. The sensitivity of the microcoulometer for the compounds studied was from 5 to 60 times greater than that of the hot-wire detector. The percentage recovery of each of the four halogenated hydrocarbons by the microcoulometer detector ranged from 75% to 95% on the silicone column and 85% to 100% on the di-*n*-decyl phthalate column at the temperatures used in this study.

Fig. 3 shows characteristic peaks for the four compounds obtained on the silicone grease and di-*n*-decyl phthalate columns at 183° and 132° with the gas chromatograph equipped with the microcoulometric detector. The silicone column separated 1,2-dibromo-3-chloropropane at 132° but it was unsatisfactory at 183°. The other three compounds were incompletely separated at 183° and almost wholly unresolved at 132°.

The di-*n*-decyl phthalate column (8 ft.) resolved the *cis*- and *trans*-1,3-dichloropropenes, and the 1,2-dichloropropane at the two temperatures studied. The 1,2-dibromo-3-chloropropane had a retention time of 90 min at 132°, but it was not eluted within a period of 240 min at 183°.

When all other conditions were constant, the compounds studied had the same elution characteristics with either helium or nitrogen as the carrier gas.

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Received June 21st, 1961

IMBIBITION AND FLOW OF COMMON SOLVENTS IN CHROMATOGRAPHY PAPER

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(Received February 18th, 1961)

The results reported here offer quantitative information on the properties of a commercial type of chromatography paper (Whatman No. 4) in the presence of various solvents.

EXPERIMENTAL

Material

Thickness of paper. According to the manufacturer, the thickness was 0.007 inch, or 0.18 mm. Our caliper measurements averaged 0.22 mm.

Weight. The weight ranged from 8.4 to 9.6 mg per cm^2. The imbibition and flow measurements given below were all corrected to a standard weight of 9.0 mg per cm^2.

Density. Strips measuring 5 × 10 cm were soaked in various solvents for three days at room temperature, then weighed in the solvents. The calculated density ranged from 1.54 to 1.57 in six common solvents; in water, it was 1.59. These differences are probably too close to the experimental error of the method to be meaningful.

Imbibition

A one-liter jar was half filled with fluid and placed under a balance. The jar was completely closed except for a small hole at the top to permit passage of a thin wire. A sheet of paper covering most of the inside wall of the jar and dipping in the fluid ensured vapor saturation. A vertical paper strip, 1 × 5 cm, was hung from the end of the wire, which was fastened to the beam of the balance. The paper strip was first completely immersed, then raised by a simple device to positions at 0.5 or 1.0 cm intervals, without otherwise disturbing the assembly. Five to nine weighings were made, each of which was the sum of three values: the weight in air of the wet part of the strip above the fluid level, the weight in fluid of the immersed part of the strip, and the surface tension along the perimeter of the strip.

The calculated weight in fluid of the immersed part was subtracted from these figures. The differences obtained, which ranged from about 50 to 200 mg, were plotted against the paper height above the fluid. A straight line could be closely fitted to them, the mean deviation of the points being about 1 mg. The slope of the line gave the weight of moist paper per cm², and the intercept with the axis of the ordinates the effect of the surface tension of the fluid.

A few minutes were usually enough to ensure constant weighings and there was no suggestion that the degree of imbibition varied much during the time needed for a series of weighings; this was shown by repeating the weighings, this time lowering the paper gradually. With water and a longer paper strip, the weight:cm² ratio remained constant as high as 15 cm above fluid level.

The results are shown in Table II. The first column gives the volume of fluid carried by 1 cm² of paper, *i.e.*, moist weight minus dry weight, divided by the density of the fluid. It is fairly clear that polar solvents produced the highest imbibition. The second column gives the observed surface tension, acting on an estimated boundary length of 2.04 cm, the perimeter of the cross section of the strip; the third column, standard values for γ from the literature. Except for water, which gave a low tension, the agreement is fairly good; the paper strip behaved as if it had been a solid plate.

TABLE II

IMBIBITION, SURFACE TENSION, AND CORRECTED FLOW RATES U_0 IN WHATMAN PAPER NO. 4

Strips 1 cm wide. Surface tensions (γ) from QUAYLE[1].

	Imbibition µl/cm²	*Surface tension obs. dynes/cm*	γ	U_0 *ml/h* 1st day	U_0 *ml/h* 2nd day	U_0 *found/* U_0 *calc.*
Water	20.0	52	72	0.228	0.231	1.00
Methanol	16.7	23	22.2	0.263	0.276	0.91
Ethanol	16.5	23	22.5	0.125	0.123	0.82
n-Butanol	13.6	25	23	0.042	0.044	0.68
Acetone	16.6	23	23	0.372	0.380	0.68
Ether	14.4	18	16.5	0.423	0.445	0.61
Chloroform	12.4	28	27.1	0.274	0.285	0.47
Carbon tetrachloride	13.8	27	26	0.162	0.173	0.44
Benzene	11.9	29	28.4	0.177	0.193	0.59
Pyridine	19.6	39	37.7	0.246	0.266	1.10

Rate of flow of water

The first step consisted of the demonstration that a strip of filter paper, bent in the shape of a siphon, actually operated like one. The apparatus used is illustrated in Fig. 1.

A strip of aluminum foil was bent at right angles in the shape of an open frame. The bottom part held a small glass cup, 3 ml in capacity. The vertical sides were provided with holes, or notches, at various heights, to hold a glass pin, which passed through two holes in the paper strip. The sawed-off bottom of a small test tube, bearing a transversal slit, was placed at the bottom of the cup to hold the end of the paper in position. The assembly was hung from a balance with the help of a thin wire and enclosed in a partially filled jar. Proper precautions (described before) were taken to avoid evaporation. It was thus easy to vary h, the difference in height between the two levels; h', the height of the paper loop above h; and a, equal to $2h' + w$, the length of the paper loop above h.

The paper strips were cut lengthwise, *i.e.*, in the machine direction; they were 0.5 cm in width and marked off with a pencil at 0.5 cm intervals. The same strip

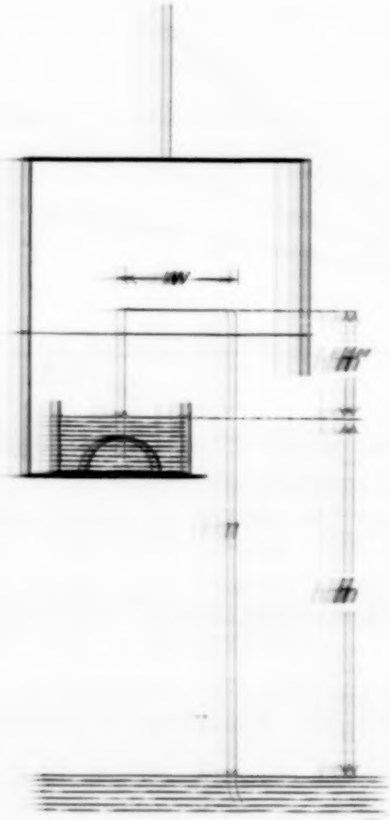

Fig. 1. Apparatus used for flow rate measurements (schematic).

was employed for each series of determinations. Weighings were made hourly in the course of a day. The rate of flow usually increased slightly in the first hour, then decreased very slowly in proportion to the drop in height in the cup. The average length of a and h, and the average temperature, were noted. The values were then corrected for the viscosity difference between the temperature of the experiment and 20°, and for the width and weight of the paper, and expressed as ml per hour per cm width.

If the system operates like a true capillary siphon, the fluid delivered per unit time, U, should be proportional to the pressure head h, and inversely proportional to the total length $a + h$:

$$\frac{U_0}{U} = \frac{h + a}{h}; \qquad \frac{1}{U} = \frac{1}{U_0} + \left(\frac{a}{U_0}\frac{1}{h}\right)$$

in which U_0 is the value of U when $a = 0$ or $h = \infty$. Plotting $1/U$ *versus* $1/h$ should give a straight line, the slope of the line should be proportional to a, and U_0 be a constant characteristic of the paper for a given solvent.

The results from a series of measurements in which h was the dependent variable are shown in Fig. 2. Theoretical expectations regarding the effect of varying the length h are confirmed by the linearity of the relation. In the first three experiments, the lines intercept the vertical axis close to $1/U = 4$. The value of U_0 would thus be about 0.25 ml per hour per cm width.

In these three experiments, the value of a calculated from the slope is 1.95, 3.5 and 9.2 respectively, in fair agreement with the actual value. The results from Expt. No. 4 were slightly discrepant and suggested that the height of loop might introduce a complicating factor.

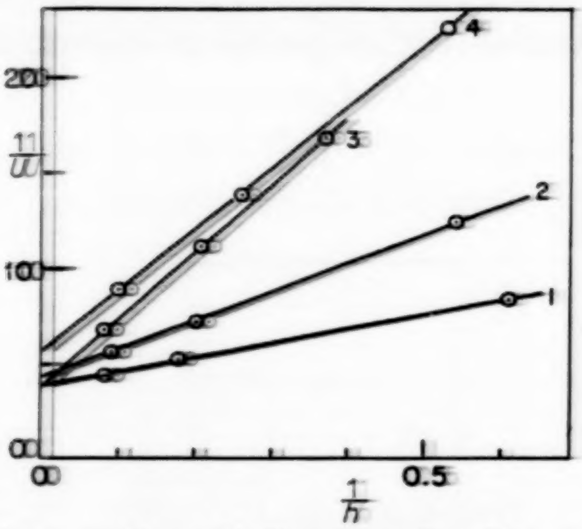

Fig. 2. Flow of water. Abscissae: reciprocal of pressure head in cm. Ordinates: reciprocal of flow in ml per hour per cm width. Expt. 1: length of loop a 2.2 cm, height h' 0.85 cm. Expt. 2: a 4.2 cm; h' 1.55 cm. Expt. 3: a 8.6 cm; h' 0.45 cm. Expt. 4: a 8.1 cm; h' 3.0 cm. Values corrected to 20°.

This point was investigated by running a series of measurements in which the pressure head h was kept constant at 2.5, while the loop, 1.5 cm in width w, varied in height h' from about 1 to 12 cm. The results (Fig. 3) show a marked deviation from theory for the higher values of a; the output was reduced by 40 % when the loop was 12 cm high.

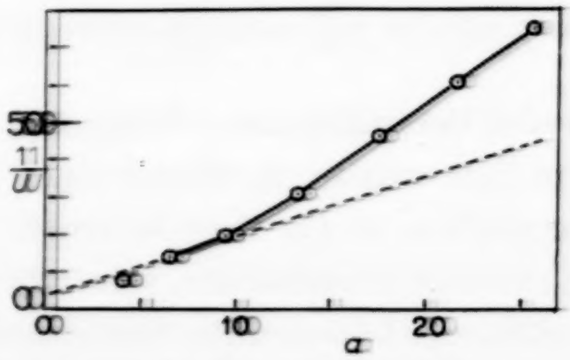

Fig. 3. Flow of water. Abscissae: length of loop in cm. Ordinates: reciprocal of flow in ml per hour per cm width. Dotted line: theoretical values for $U_0 = 0.25$.

Rate of flow of organic fluids

The experiments with water, illustrated in Fig. 2, were repeated with methanol. The results were essentially the same, *i.e.*, the same close linear relation was obtained between $1/U$ and $1/h$; however, the intercept of the lines with the axis of ordinates showed a wider scatter. Therefore, in order to minimize the danger of errors due to the loop, the latter was made as short, the total length as long, as conveniently possible. The measurements were as follows: $h' = 0.8$ to 1.3 cm; $w = 1.5$ cm; $a = 2\,h' + w =$

3.5 cm approximately; $h = 33$ cm. The paper strips were 1 cm in width and cut in succession out of the same sheet.

Weighings were made at hourly intervals for one day, the apparatus left standing overnight, the cup refilled, and weighings made again the second day. They were corrected as above to yield U; U_0 was then equal to $U \times \frac{33 + 3.5}{33}$. These values are given in Table I (4th and 5th columns). The flow was usually slightly faster on the second day. The last column shows the ratio between observed flow (average of first and second day) and that expected if the differences in rates between water and other fluids had been solely due to differences in density and viscosity. There is a clear relation between rate of flow and polarity.

DISCUSSION

The purpose of the present study was to estimate the amounts of various fluids imbibed by Whatman paper No. 4 and the rate of flow of these fluids under the effect of gravity. The nature of the work precluded high accuracy. The results are reported primarily for their order of magnitude and their comparative significance, since no such information appears to exist in the literature.

CASSIDY[2] has reviewed previous work in this field, devoted mostly to the study of capillary ascent of fluids in paper. He remarks about the "extremely complicated system and many variables" offered by filter paper. In view of the difficulty of defining a standard state of the material to which measurements might be referred and of deciding by what preparatory means such a state should be reached, it seemed warranted here, especially considering the preliminary nature of the results, to use the paper as it came from the manufacturer.

It must be stated that the characteristics of the paper in the presence of the fluids appeared very stable. It was not observed that preliminary treatment such as drying at 100°, exposure to vapors or contact with any of the fluids had a permanent effect on the properties of the material. Slow changes are, of course, not ruled out and the results are considered significant only within the limits of the experimental conditions.

The measurements of imbibition require little comment. It is unlikely, in view of the close linear relation between the height and the weight of the wet strips, that an important "weight profile" existed, such as is observed during the capillary ascent of fluid in paper[2,3]*. The distribution of fluid seems to have been even up to a height of at least 5 cm, the polar fluids being definitely more strongly imbibed.

The measurements of flow offered unexpected difficulties. Attempts to make the paper start from the bottom of a container were fruitless because of the impossibility of devising a satisfactory joint. Hence the need for a "loop" and for the demonstration that the system acted like a true siphon. This was given, within limits, for water.

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* In the course of this work, it was noticed that the equation describing initial fluid rise in capillary tubes, as given by BIKERMAN[4], differs from that of LIGENZA AND BERNSTEIN[5] by the factor 2. Dr. W. R. THOMPSON, who was kind enough to read this manuscript, has pointed out (one-page note No. 41759, available in Bruning reproduction) the error made by the latter authors.

Furthermore, the close linearity of the relation between $1/U$ and $1/h$ suggested that, with water and methanol at least, there was no important weight profile in the descending part of the strip.

The loop effect was not studied with other fluids. In the flow measurements reported in Table I, conditions were such that this effect cannot have been considerable, so that the values given for U_0 come probably close to what they would be if it were possible to reduce the loop to zero length. In the last column, the relation between polarity and the ratio of observed to expected flow is quite striking.

In the early part of the work, flow measurements were made on strips 0.5 cm in width, and these gave usually higher values than those reported here. As the strip is widened, the rate would probably tend to a minimum per cm width; as the strip is lengthened, the rate tends to a maximum. It would thus represent a sort of constant of the paper for each fluid studied.

The results may be compared with those of KRESS AND BIALKOWSKY[6] on paper swelling: 90% by volume in water, 62% in methanol, 40% in ethanol, 4% in butanol. In the present instance, one cm^2 of paper had a volume of 22 mm^3, of which 5.7 mm^3 was occupied by 9 mg of cellulose and 16.3 mm^3 by air. Bound fluid would thus have occupied volumes of 5.1, 3.5, 2.3, and 0.2 mm^3 respectively, leaving (after subtraction from the first column in Table I) 16.9, 13.2, 14.2, and 13.4 mm^3 of free fluid in the open spaces. These figures only help to visualize the problem and are too imprecise to warrant much speculation. If polar fluids produce not only greater swelling but also relatively more rapid flow, this might be ascribed to some change in the shape of the fibers; becoming less ribbon-like and more cylindrical, these might cause the open spaces to be less ramified and thus offer less resistance to the movement of fluid. Differences in relative flow rates of 1 to 2, such as were observed here, could be due to differences in the capillarly bed too small to be correlated with the information yielded by measurements of imbibition.

SUMMARY

The properties of a commercial chromatography paper (Whatman No. 4) were studied with water and nine organic fluids. Imbibition was greater with the more polar fluids and ranged from 22.0 mm^3/cm^2 for water to 11.9 mm^3/cm^2 for benzene. Measurements of flow rates under the influence of gravity showed that, the more polar the fluid, the smaller the resistance to flow offered by the paper. The rate of water was 0.23 ml per hour per cm width at 20°.

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PAPER CHROMATOGRAPHY OF MIXTURES OF PHENOLIC COMPOUNDS

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(Received January 31st, 1961)

INTRODUCTION

Chromatography could greatly simplify the analysis of the degradation products of hardwood lignin, provided the use of a single eluent could give, on the sheets, a good separation of the main degradation products, *viz.*: vanillic, *p*-hydroxybenzoic and syringic aldehydes and acids, and ferulic and *p*-coumaric acids.

Many authors have published papers on the qualitative and quantitative analysis of phenolic compounds by paper chromatography[1,2]. In the generally employed technique[2,4–7] two or more eluents are used, and even recently, in a series of papers on the alkaline hydrolysis of hardwoods, PEARL and co-workers[8–11] were able to separate the above-mentioned compounds by using as many as four eluents.

If we consider REIO's[12] determination of the R_F values of 450 compounds, we find that none of the six eluents used by him gives a complete separation of all the eight products; to obtain a separation good enough for quantitative purposes, the R_F values must differ by at least 0.04. We have succeeded in developing a chromatographic technique with which a mixture of these compounds can be separated with a single eluent for qualitative and quantitative evaluation. At the end of this paper the R_F values of 34 pure phenolic compounds obtained with our technique are given.

EXPERIMENTAL

In our experiments, an hermetically sealed double-walled chromatographic tank was used, generally at room temperature (20 ± 2°); for experiments at other temperatures, the tank was placed in a thermostatically controlled oven. The sheets were cut from Whatman papers, across[1] the fibre direction.

Descending elution was employed; the distance of the starting line from the edge was 10 cm. Ethanolic solutions of the phenolic compounds with concentrations of 10 $\mu g/\mu l$ were applied on the starting line as spots of 2 μl for each component, either pure or in a mixture.

The eluent was freshly prepared by shaking equal volumes of *n*-butanol and 2% aqueous ammonia for 10 min; the aqueous phase was separated and put at the bottom of the tank.

Paper and eluent

Of the four basic Whatman papers Nos. 1, 7, 20, 54, cut across the fibre direction, we found that Whatman No. 7 is the most suitable for the separation of single compounds; dense, uniform, fairly round spots without trails or beards were obtained, with an elution speed of 33 to 35 cm in 15 h. However, separation of the substances from a mixture was impossible; the three aldehydes were not resolved, nor were vanillic and *p*-hydroxybenzoic acid.

Various authors have obtained better results by using buffered paper. Other variables being kept constant, the R_F values are more reproducible and the separation is somewhat better when buffered paper is used, due to complex formation or to the influence of the pH value at which the paper has been conditioned.

McFARREN[13] found that the molarity of the buffer is of no consequence, whereas the R_F values are sensitive to the particular salt used in preparing the buffer solution. We found, for instance, that at the same pH value the results were different when phosphate-buffered or borate-buffered papers were used, probably because phenols can react with the boric ion.

GARDON AND LEOPOLD[14] applied McFARREN's technique to the study of the products of lignin oxidation; the R_F's were determined for each compound on a series of papers buffered at different pH values. Since the R_F's of ionisable substances are sensitive to pH variations, a diagram of R_F *versus* pH will show the values of the pH at which the separation of the different compounds is optimal.

We impregnated Whatman No. 7 sheets with mixtures of H_3BO_3 and 1 N NaOH, in a pH range from 3.5 to 9.2. It was found that care is needed to obtain a uniformly impregnated sheet, which is necessary to prevent the formation of irregular spots. A sufficiently uniform impregnation was never obtained by spraying, so we resorted to immersing the sheet in the appropriate bath, removing the excess of solution by gently pressing the sheet between two filter papers, and then allowing it to dry in air.

At this point we also changed the eluent, going over to *n*-butanol saturated with the same solutions that were used to impregnate the sheets, in order to prevent pH gradients along the sheets. Otherwise the mobility of the spots would be so high that separation and identification would be impaired.

In Fig. 1 the diagram of R_F *versus* pH for the eight pure compounds studied is shown; the hydroxyaldehydes are best separated at pH 9.3 and the phenolic acids at pH 8.0. On repeating the experiment with a mixture of the various compounds, no complete separation could be obtained whatever pH value was used. This might be due to an interaction of the molecules of the different phenolic compounds, so that their mobility is different from that of the pure compounds.

We repeated the experiments with pure products on paper impregnated at four pH values, while 10 ml of a 10 % NH_3 solution was placed at the bottom of the chromatographic tank, together with the aqueous phase of the eluent (*n*-butanol saturated with the impregnating solution). Fig. 2 shows a diagram of the R_F values *versus* pH; it can be seen that the separation of the phenolic compounds is markedly improved. We also found that the pH values of the sheets which varied from 3.5 to 8.5 at the beginning of the experiment, were all 8.6 at the end of the experiment. In the case of papers impregnated at pH 9.3, however, not only the pH remained the same throughout the whole experiment, but also the relative positions of the spots were almost unaltered; at this value of the pH, the presence of NH_3 vapour in the vessel is of no consequence.

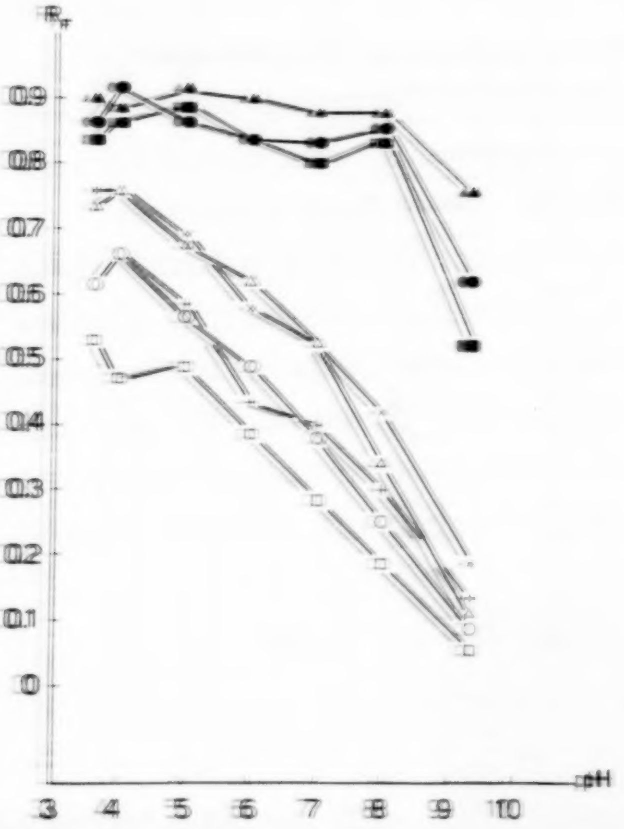

Fig. 1. R_F *versus* pH values. ● Vanillin; ■ Syringaldehyde; ▲ *p*-Hydroxybenzaldehyde; ○ Vanillic acid; △ *p*-Hydroxybenzoic acid; □ Syringic acid; + Ferulic acid; × *p*-Coumaric acid.

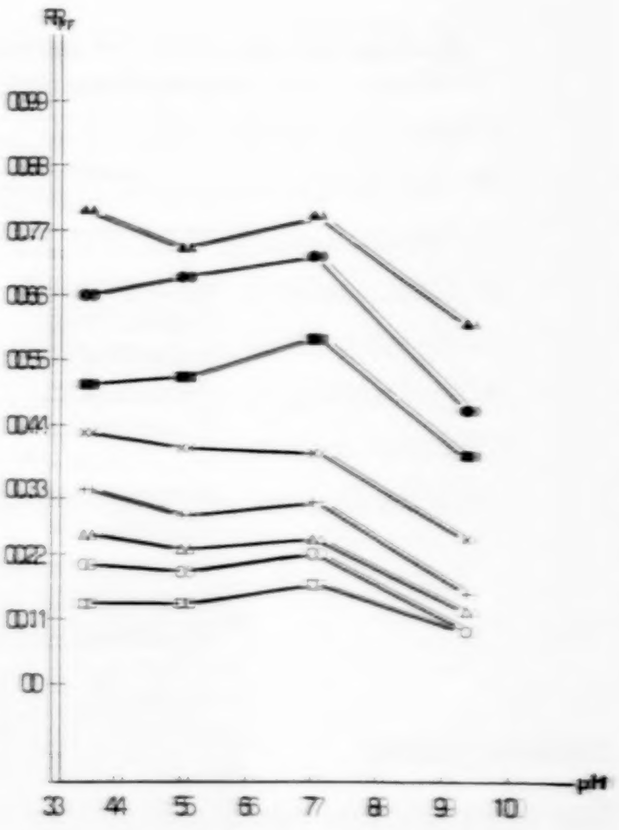

Fig. 2. R_F *versus* pH values, in the presence of NH_3 vapours. ● Vanillin; ■ Syringaldehyde; ▲ *p*-Hydroxybenzaldehyde; ○ Vanillic acid; △ *p*-Hydroxybenzoic acid; □ Syringic acid; + Ferulic acid; × *p*-Coumaric acid.

On the other hand, the separation of the mixture was appreciably improved by the presence of ammonia vapours at all pH values. It is not necessary to buffer the paper, a simple impregnation with boric acid is sufficient; it is also not necessary to saturate the eluent with the impregnating solution.

Accordingly, in all successive experiments, the sheets were impregnated with a H_3BO_3 solution saturated at 20°, and the eluent was *n*-butanol saturated with a 2% NH_3 solution, the aqueous phase of which was placed at the bottom of the vessel.

At the end of the elution, the pH of the sheet was uniformly 8.6 from the starting line to the eluent front, and from there to the edge of the paper. This procedure has given the best separation of a mixture of ten phenolic compounds, whose R_F values are given in Table I.

The chromatographic separation of a mixture of phenolic compounds by this technique is very sensitive to the concentration of ammonia in the aqueous solution used to prepare the eluent. Increasing the concentration of ammonia produces a slowing down of the phenolic acids and hydroxyaldehydes: at an ammonia concentration higher than 5% the separation is seriously impaired. On the other hand, if the ammonia concentration is raised to 10%, the separation of those phenolic compounds that run with the eluent at lower ammonia concentrations is improved. An R_F of 0.88 is obtained for 2,6-dimethoxyphenol and one of 0.76 for resorcinol, instead of the values 0.86 and 0.81 respectively, as given in Table I, and hence the separation is much better.

TABLE I

R_F VALUES OBTAINED ON CHROMATOGRAPHY OF A MIXTURE OF TEN PHENOLIC COMPOUNDS, AND THE EFFECT OF TEMPERATURE

Phenolic compounds	*R_F values*	
	Tank at 20°	*Tank at 35°*
Syringic acid	0.16	0.15
Vanillic acid	0.20	0.19
p-Hydroxybenzoic acid	0.24	0.23
Ferulic acid	0.32	0.33
p-Coumaric acid	0.39	0.40
Syringaldehyde	0.52	0.52
Vanillin	0.62	0.64
Hydroxybenzaldehyde	0.72	0.71
Resorcinol	0.81	—
2,6-Dimethoxyphenol	0.86	—

Ambient factors

The temperature variations in the laboratory were ± 2°; with our final technique, the R_F values of the various compounds were always constant. The relative positions of the spots of the compounds are the same, although their absolute positions may change, but the variations in the R_F values are never higher than 0.02. Some experiments were made at 35°, at which the solvent speed was somewhat increased and consequently also the mobilities of the spots, but the R_F's were unchanged, as is shown in the third column of Table I.

The saturation of the atmosphere of the chromatographic tank and the equilibration of the sheet are much more important. Only the aqueous phase should be put at the bottom of the tank; an excess of ammonia or of the phase rich in organic solvent gives elongated spots.

If the sheets are left for a long time in the tank before starting the elution, very diffuse spots with overlapping trails are produced. We found that good spots are obtained if the paper is allowed to equilibrate for one hour in the vessel.

Spraying solutions

All the classical developers for phenolic compounds[1,2], as well as some new ones, were tested and finally the following techniques were adopted:

(1) *Mäule's test*. The chromatogram is first dried, then exposed for ten minutes to chlorine vapours, and finally sprayed with a 10% Na_2SO_3 solution. Spots of syringic acid or aldehyde assume a cherry-red colour. It was found that on examination with U.V. light some compounds show characteristic yellow or blue spots.

(2) *Other tests.* The chromatogram is subjected to the following treatment:

(a) U.V. examination before and after exposure to ammonia vapours.

(b) Development of the phenolic acids: the sheet is first sprayed with a solution of diazotized sulphanilic acid prepared according to BLOCK[15] and dried in air. Subsequently, it is exposed to ammonia vapours. At this moment, coloured spots appear, but the colours are not brilliant and not fast enough; they fade in a short time.

(c) We then introduced a second spray, this time with a solution of diazotized *p*-nitroaniline prepared according to BRAY[16]; this changes the colours, enhances their brilliance and makes them completely fast even after weeks of exposure to air.

The sheet is dried at 50°. The hues of the spots are characteristic for the various acids. Care has to be exercised when carrying out this last spray, in order to prevent soaking of the paper, otherwise a yellowish background appears and the sheets become brittle when dried, due to the action of the hydrochloric acid present in the solution. Of paramount importance in this technique is the exposure of the sheet to ammonia vapour between the two sprayings with the diazotized solutions; if this stage is omitted, the benefits obtained by spraying with the second solution are lost.

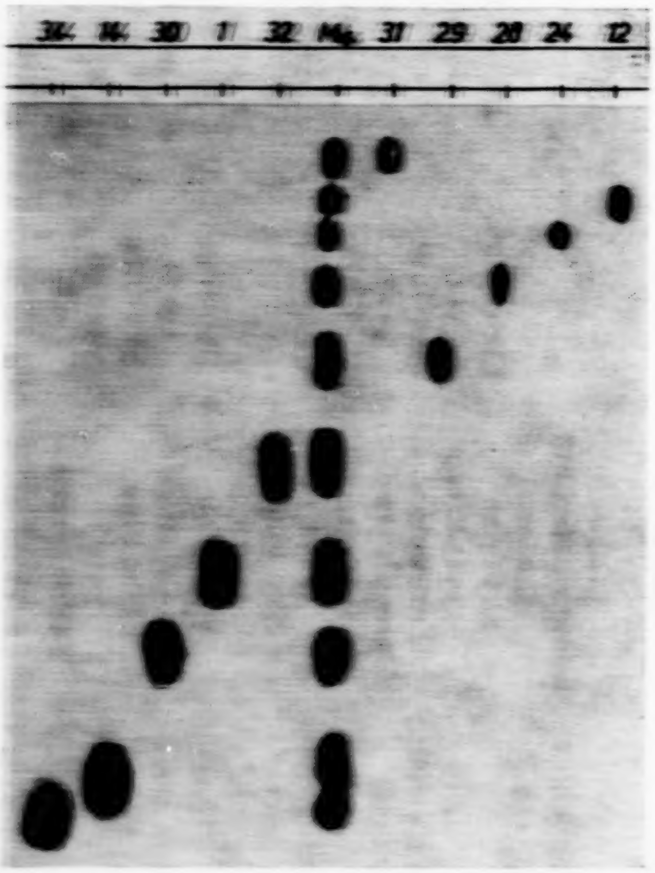

Fig. 3. Chromatographic separation of a mixture of ten phenolic compounds. (12) Vanillic acid; (24) *p*-Hydroxybenzoic acid; (28) Ferulic acid; (29) *p*-Coumaric acid; (31) Syringic acid; (32) Syringaldehyde; (1) Vanillin; (30) *p*-Hydroxybenzaldehyde; (14) Resorcinol; (34) 2,6-Dimethoxyphenol.

(d) Development of the hydroxyaldehydes: the dried sheet is sprayed with a solution obtained by mixing equal volumes of a 0.1 *M* benzidine solution in ethanol and 1 *N* hydrochloric acid. The sheet is then dried in an oven at 50°. The colours obtained with this technique are more brilliant than those obtained by spraying with 2,4-dinitrophenylhydrazine. Furthermore, vanillin and syringaldehyde give different colours.

TABLE II

R_F VALUES AND IDENTIFICATION OF 34 PURE PHENOLIC COMPOUNDS*

Compounds	R_F	*Mäule test*	*Mäule test Ultraviolet*	*U.V.*	*U.V. after exposure to NH_3 vapours*	*Diazotised sulphanilic acid spray*	*Diazotized sulphanilic acid and p-nitroaniline sprays*	*Benzidine spray*
Gallic acid	0.01	Magenta 27142	—	—	—	Straw-yellow 33793	Straw-yellow 33793	—
Syringic acid	0.16	Cherry-red 22356	Water-green 24554	—	—	Cherry-red 22356	Crimson lake 21158	—
Vanillic acid	0.20	—	Lemon-cadmium 33814	—	—	Middle chrome 33538	Rose-pink 32356	—
p-Hydroxybenzoic acid	0.24	—	Sky-blue 35231	—	—	Deep cadmium 13432	Gold 33793	—
Catechol	0.29	Zinc yellow 33434	Lemon-cadmium 33814	—	—	Lemon-cadmium 33814	Naples yellow 10371	—
Ferulic acid	0.32	—	Sky-blue 35231	Light blue 15123	Light blue 15123	Pink madder lake 31433	Light violet 37144	—
Sinapic acid	0.35	Cherry-red 22356	Turquoise-green 15123	Sky-blue 35231	Sky-blue 35231	Cherry-red 22356	Blue-grey 36173	—
Vanillil	0.35	—	Yellow-green 34552	—	Gold 33793	Gold 33793	—	—
p-Coumaric acid	0.39	—	Sky-blue 35231	—	Sky-blue 35231	Pale vermilion 22276	Magenta 27142	—
3,4-Dimethoxycinnamic acid	0.42	—	Sky-blue 35231	Sky-blue 35231	Sky-blue 35231	—	—	—
o-Coumaric acid	0.45	—	Light blue 15123	Sky-blue 35231	Sky-blue 35231	Middle chrome 33538	Hazel 12648	—
Veratric acid	0.46	—	Yellow-green 34552	—	—	—	—	—
Syringaldehyde	0.50	Cherry-red 22356	Water-green 24554	—	Light blue 15123	—	—	Orange chrome 32246
p-Anisic acid	0.55	—	Sky-blue 35231	—	—	—	—	—
Vanillin	0.60	—	Lemon-cadmium 33814	—	Light blue 15123	—	—	Middle chrome 33538
Trimethylgallic acid	0.61	Magenta 27142	Water-green 34554	Ultramarine 25414	Ultramarine 25414	—	—	—
Salicylic acid	0.69	—	Sky-blue 35231	Sky-blue 35231	Sky-blue 35231	Deep cadmium 13432	Gold 33793	—
2,4-Dimethoxybenzaldehyde	0.78	—	—	Ultramarine 25414	Ultramarine 25414	—	—	Middle chrome 33538
Acetovanillone	0.78	Pale yellow 23605	Sky-blue 35231	Sky-blue 35231	Dark blue 15182	Flesh-pink 21575	Gold 33793	—

p-Hydroxybenzaldehyde	0.71	—	Sky-blue 35231	—	Dark brown 10080	—	—	Primrose-yellow 23655
Resacetophenone	0.71	—	Yellow-green 24552	Gold 33793	Rose-pink 32356	Gold 33793	Gold 33793	—
p-Methoxycinnamic acid	0.74	—	Sky-blue 35231	Sky-blue 35231	Sky-blue 35231	—	—	—
Vanillyl alcohol	0.80	—	Lemon-cadmium 33814	—	—	Middle chrome 33538	Rose-pink 32356	—
2,4-Hydroxyacetophenone	0.81	—	Sky-blue 35231	Water-green 24554	Water-green 24554	Pale yellow 33481	Gold 33793	—
Resorcinol	0.81	Zinc yellow 33434	Sky-blue 35231	Sky-blue 35231	Sky-blue 35231	Straw-yellow 33793	Deep cadium 13432	—
Veratraldehyde	0.85	—	Ultramarine 25414	Water-green 24554	Water-green 24554	—	—	Middle chrome 33538
2,6-Dimethoxyphenol	0.86	Cherry-red 31136	Yellow-green 34552	—	—	Orange chrome 32246	Geranium lake 12197	—
Hydroquinone	0.89	—	—	—	—	—	Flesh-pink 21575	—
Isoeugenol	0.89	Pale yellow 23695	Lemon-cadmium 33814	—	—	Flesh-pink 21575	Flesh-pink 21575	Primrose-yellow 23655
Eugenol	0.92	Magenta 27142	Sky-blue 35231	—	—	Flesh-pink 21575	Flesh-pink 21575	—
4,4'-Dihydroxybiphenyl	0.92	Pale yellow	Sky-blue 35231	Sky-blue 35231	Sky-blue 35231	—	—	—
Dihydroisoeugenol	0.95	—	Sky-blue 35231	—	—	Flesh-pink 21575	Flesh-pink 21575	—
Guaiacol	0.95	—	Water-green 34554	—	—	Flesh-pink 21575	Flesh-pink 21575	—
Coumarin	0.95	—	Sky-blue 35231	—	—	Pale yellow 23695	—	—
Phloroglucinol	streak	—	Sky-blue 35231	—	—	Lemon-cadmium 33814	Zinc yellow 33434	—
Pyrogallol	streak	Cherry-red 22356	Yellow-green 34552	—	—	Raw sienna 33481	Hazel 12648	—

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[*] Colour recording of the spots of the chromatograms was performed according to Federal Standard No. 595, March 1, 1956 (U.S.A.).

R_F values of some phenolic compounds

Fig. 3 shows a chromatogram of the separation of a mixture of ten compounds, and Table II gives the R_F values for 34 pure phenolic compounds.

SUMMARY

A qualitative method of paper chromatography for phenolic compounds is discussed. Whatman No. 7 paper was impregnated with a saturated solution of boric acid. The solvent used was the organic phase of *n*-butanol saturated with 2% NH_4OH. The time of development was 15 hours.

Beside the classical methods of spot detection, two new processes were employed: (a) ultra-violet examination of the chromatogram after Maule's test; (b) spraying with a solution of diazotized sulphanilic acid, exposure to ammonia vapours followed by spraying with a solution of diazotized *p*-nitroaniline.

A table of R_F values of 34 pure phenolic compounds is given, together with methods for their identification.

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PAPER CHROMATOGRAPHY OF PHENOLS

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(Received March 29th, 1961)

The separation and identification of free phenols by paper chromatography have been studied extensively during the last 15 years, and a very great number of solvents have been proposed. The literature has been reviewed up to 1956 mainly by BLOCK AND ZWEIG[1], DIETRICH[2], LEDERER AND LEDERER[3] and HAIS AND MACEK[4]. Of the more recent work in this field the publications should be mentioned of REIO[5], COULSON AND EVANS[6] and PRIDHAM[7], who proposed a great many solvent systems, using plain or buffer-treated paper. KUNG-TSUNG WANG[8] uses a polyamide-impregnated paper and solvents such as *n*-butanol, ethyl acetate, toluene, benzene, cyclohexane, cyclohexene or *n*-hexane. MACÁK AND KUBEŠ[9] recently described a method using cyclohexane as the mobile phase on paper impregnated with formamide.

In connection with extensive analytical work on phenols in natural products, we needed a paper chromatographic method that would give a precise and good separation. In our hands many of the solvent systems described in the literature failed to give the results desired. Stimulated by our previous good results with two-phase solvent systems[10,11] and by the comprehensive work of GASPARIČ AND VEČEŘA[12], we devised a simple method using dimethylformamide as the stationary phase and a mixture of cyclohexane and ethyl acetate as the mobile phase. A variety of free monophenolic compounds have been separated satisfactorily.

GENERAL PROCEDURE

Sheets of Schleicher & Schüll paper No. 2043b Mgl (or Macherey, Nagel & Co., Düren, No. 2214), 19 × 54 cm, were impregnated by drawing them through a mixture of dimethylformamide–acetone (75:25, v/v); then they were lightly pressed between sheets of filter paper and hung in the air for a short time to evaporate the acetone. The phenols to be chromatographed were dissolved in chloroform (1 mg per ml) and 3–5 μl of the solution was placed, in the usual way by means of a micropipette, on the starting line, 12 cm from one end of a sheet prepared as described above (6 substances on each sheet).

After 15 minutes equilibration of the sheet in the chromatography chamber, the mobile phase, consisting of a mixture of cyclohexane–ethyl acetate (5:1, v/v), which had been previously saturated by agitating with a mixture of dimethylformamide–water (5:2, v/v), was poured into the trough. It is possible to increase the R_F values by increasing the quantity of ethyl acetate in the mobile phase. The proportion cyclohexane–ethyl acetate should not exceed 5:2 v/v, other experimental conditions remaining unchanged. The chromatograms were run by the descending method at 25°, the solvent front travelling about 35 cm from the starting line in 3 1/4–3 1/2 hours. The paper sheets are kept and prepared in a laboratory with a constant relative humidity of 45%.

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* Director of Research: Dr. M. STOLL.

As already observed during our earlier work with similar two-phase solvent systems[10,11], correct saturation of the atmosphere in the chromatography chamber is essential for good separation and round spots (no "tailing"). The two side walls (20 × 50 cm) of a rectangular glass jar (20 × 30 cm and 50 cm high) were lined with filter papers dipping into crystallizing dishes containing dimethylformamide–water (5:2, v/v). The lining papers were completely moistened every time a new chromatogram was placed in the jar. On the bottom of the chromatography chamber two small crystallizing dishes containing the mobile phase were placed.

The finished chromatogram was dried for 10 min at 40° and in most instances sprayed with diazotized sulphanilic acid and sodium carbonate as described in the literature[13,1]. Diazotized *p*-nitroaniline and ferric chloride solution as described[1], were also used as spraying agents in some cases. Examination of the chromatogram in ultraviolet light may sometimes help in the identification (fluorescence).

RESULTS AND DISCUSSION

In spite of very constant experimental conditions, the R_F values in a two-phase solvent system as used here, may show small variations (up to 0.05 units) from one chromatogram to another, the principal cause being probably due to differences in the impregnation grade.

The R_F values in Table I are relative values obtained by running a special reference mixture of five substances (R_F values 0.12–0.70) on every chromatogram. The R_F values of the reference mixture itself were determined as the average value of more than 30 chromatograms. The R_F of each substance in the list is an average value obtained from 5 to 20 test chromatograms, each test chromatogram being in correct relationship to the values of the reference mixture.

As shown in the photographs (Fig. 1), the method gives sharp round spots without "tailing" and a good separation of a great many mono-phenols.

A difference of 0.05 in the R_F values is necessary to obtain separation of two compounds. In general, substances chromatographed separately and in mixtures show the same R_F values. The sensitivity of the method is satisfactory. With diazotized sulphanilic acid, 0.1 μg of substance could still be detected.

The method is based on a partition between a strongly polar stationary phase (dimethylformamide) and a less polar mobile phase (cyclohexane–ethyl acetate).

TABLE I

R_F VALUES AND COLOUR REACTIONS OF VARIOUS MONOPHENOLIC COMPOUNDS

Descending paper chromatography at 25°. Paper: Schleicher & Schüll, No. 2043 b Mgl, impregnated with dimethylformamide–acetone (75:25, v/v); mobile phase: cyclohexane–ethyl acetate (5:1, v/v), saturated with dimethylformamide–water (5:2, v/v). Spraying agent: diazotised sulphanilic acid, then 20 % Na_2CO_3[13].

Compound	*R_F value*	*Colour*
p-Aminophenol	0	grey-blue
m-Aminophenol	0	yellow
o-Aminophenol	0.05	light brown
o-Hydroxybenzyl alcohol	0.06	yellow
Vanillin (4-Hydroxy-3-methoxybenzaldehyde)	0.10	light brown
4-(*p*-Hydroxyphenyl)-butan-2-one	0.12	rose
4-(*m*-Hydroxyphenyl)-butan-2-one	0.14	yellow-orange
p-Nitrophenol	0.18	lemon-yellow
4-(*p*-Hydroxyphenyl)-butan-3-one	0.18	rose
2-Hydroxy-1,3-dimethoxybenzene (Pyrogallol 1,3-dimethyl ether)	0.19	red
Hydroquinone monomethyl ether (1-Hydroxy-4-methoxybenzene)	0.19	violet
Ethylvanillin (4-Hydroxy-3-ethoxybenzaldehyde)	0.21	pale brown
5-(*p*-Hydroxyphenyl)-pentan-2-one	0.25	rose
Phenol	0.34	yellow
Guaiacol (1-Hydroxy-2-methoxybenzene)	0.35	orange
4-(*o*-Hydroxyphenyl)-butan-2-one	0.35	orange
o-Vanillin (2-Hydroxy-3-methoxybenzaldehyde)	0.39	yellow
p-Phenylphenol (4-Hydroxydiphenyl)	0.40	dark violet
Vinylguaiacol (1-Hydroxy-2-methoxy-4-vinylbenzene)	0.41	rose-violet
m-Cresol (*m*-Methylphenol)	0.41	yellow
p-Cresol (*p*-Methylphenol)	0.42	rose
3,4-Dimethylphenol (4-Hydroxy-1,2-dimethylbenzene)	0.45	rose
Creosol (2-Methoxy-4-methylphenol)	0.46	rose-light violet
p-Benzylphenol (4-Hydroxydiphenylmethane)	0.48	pink, pale
o-Cresol (*o*-Methylphenol)	0.49	yellow-orange
3,5-Dimethylphenol (5-Hydroxy-1,3-dimethylbenzene)	0.50	yellow
o-Phenylphenol (2-Hydroxydiphenyl)	0.51	orange
Isoeugenol (4-Hydroxy-3-methoxy-1-propenylbenzene)	0.52	violet
p-Ethylphenol (4-Hydroxy-1-ethylbenzene)	0.54	rose-violet
2,3-Dimethylphenol (3-Hydroxy-1,2-dimethylbenzene)	0.56	orange
2,5-Dimethylphenol (2-Hydroxy-1,4-dimethylbenzene)	0.57	orange
2,4-Dimethylphenol (4-Hydroxy-1,3-dimethylbenzene)	0.59	violet
Eugenol (4-Hydroxy-3-methoxy-1-allylbenzene)	0.60	violet
o-Nitrophenol	0.60	pale yellow
4-Hydroxy-3-methoxy-1-ethylbenzene (*p*-Ethylguaiacol)	0.61	violet
2,6-Dimethylphenol (2-Hydroxy-1,3-dimethylbenzene)	0.65	orange
2-Ethoxy-4-methylphenol	0.66	violet
2-Hydroxy-1-ethoxy-4-propenylbenzene	0.68	orange
p-*tert.*-Butylphenol (4-Hydroxy-1-*tert.*-butylbenzene)	0.70	pink, pale
Butylhydroxyanisole (2- and 3-*tert.*-Butyl-4-methoxyphenol)	0.74	pink, pale
Carvacrol (2-Hydroxy-1-methyl-4-isopropylbenzene)	0.76	orange
Thymol (3-Hydroxy-1-methyl-4-isopropylbenzene)	0.79	orange
o-*tert.*-Butylphenol (2-Hydroxy-1-*tert.*-butylbenzene)	0.81	orange

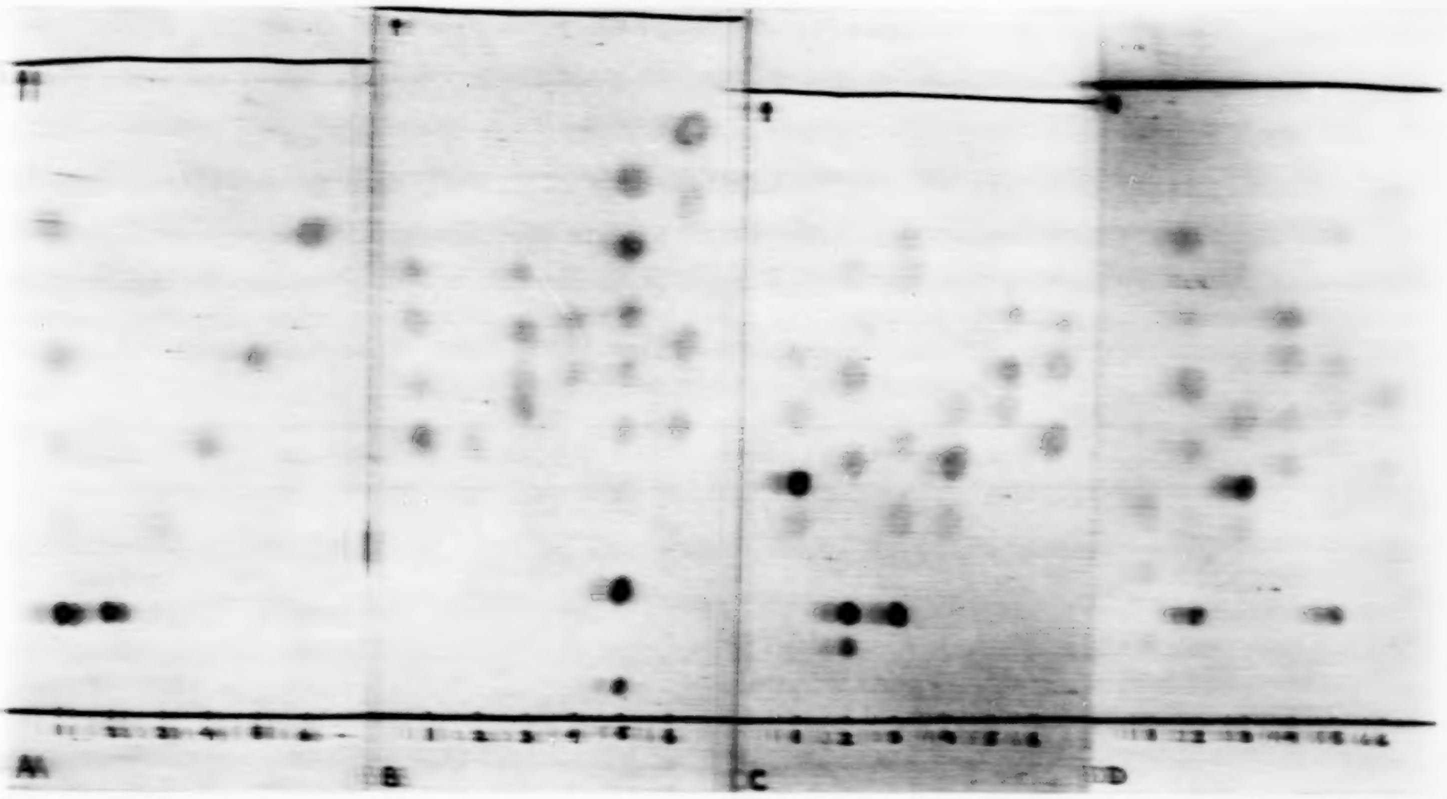


Fig. 1. Photographs of mono-phenolic compounds (sprayed with diazotized sulphanilic acid and sodium carbonate).

A. (1) 2-Hydroxy-1,3-dimethoxybenzene, phenol, creosol, eugenol, thymol; (2) 2-Hydroxy-1,3-dimethoxybenzene; (3) Phenol; (4) Creosol; (5) Eugenol; (6) Thymol.

B. (1) *p*- and *o*-Cresol, 2,4- and 2,6-dimethylphenol; (2) *m*-Cresol; (3) 3,4-; 3,5-; 2,3- and 2,6-Dimethylphenol; (4) Isoeugenol, eugenol; (5) *o*-Aminophenol, hydroquinone monomethyl ether, phenol, *p*-phenylphenol, *p*-benzylphenol, eugenol, 2-hydroxy-1-ethoxy-4-propenylbenzene, carvacrol; (6) *p*- and *o*-Phenylphenol, *p*- and *o*-*tert.*-butylphenol.

C. (1) Phenol, vinylguaiacol, *o*-cresol, eugenol; (2) 4-(*p*-Hydroxyphenyl)-butan-2-one, 2-hydroxy-1,3-dimethoxybenzene, *p*-phenylphenol, *p*-ethylphenol, *p*-*tert.*-butylphenol; (3) 2-Hydroxy-1,3-dimethoxybenzene, phenol, creosol, *p*-*tert.*-butylphenol, carvacrol; (4) Phenol, *p*- and *o*-cresol; (5) 3,5-; 2,3- and 2,6-Dimethylphenol; (6) 3,4-; 2,4- and 2,6-Dimethylphenol.

D. (1) 4-(*m*-Hydroxyphenyl)-butan-2-one, 5-(*p*-hydroxyphenyl)-pentan-2-one, 4-(*o*-hydroxyphenyl)-butan-2-one; (2) 2-Hydroxy-1,3-dimethoxybenzene, phenol, creosol, *p*-ethylphenol, 2-ethoxy-4-methylphenol, carvacrol; (3) Guaiacol, vinylguaiacol, *p*-benzylphenol, 4-hydroxy-3-methoxy-1-ethylbenzene, butylhydroxyanisole; (4) *p*- and *o*-Cresol, 2,4- and 2,6-dimethylphenol; (5) 2-Hydroxy-1,3-dimethoxybenzene, phenol, creosol, isoeugenol, eugenol, thymol; (6) *p*- and *o*-Phenylphenol, *p*- and *o*-*tert.*-butylphenol.

A sufficiently great difference in polarity (acidity) between two compounds is the condition for their separation on the paper chromatogram.

These experimental results show an interesting relationship between R_F value and acidity (pK_a). To a certain extent, this relationship permits one to predict the structure from the R_F value. The influence of the substituents of the phenols is due partly to their *nature*, and partly to their *position*. The chief effects are: (a) inductive effect, (b) steric hindrance, (c) hyperconjugation, (d) mesomeric effect. For general information on these effects, we refer the reader to the literature[14].

Some of these relationships are illustrated in Table II.

In addition to the relationships shown in Table II, some interesting facts are briefly discussed below.

Cresols

The replacement of one hydrogen in the phenol nucleus by the electron-*donating* methyl group (+ *I* substituent[14]) decreases the acidity of the phenolic hydroxyl

TABLE III

RELATIONSHIPS BETWEEN ACID STRENGTH (pK_a), R_F VALUE, ELECTRONIC AND STERIC EFFECTS, OF SOME SUBSTITUTED MONOPHENOLIC COMPOUNDS

Substances	pK_a*	R_F**	*Principal effects (relative strength)*		
			Inductive	*Steric*	*Hyperconjugation*
Phenol	9.98[15]	0.34			
m-Cresol	10.08[15]	0.41	—	—	—
p-Cresol	10.10[16]	0.42	+	—	+
o-Cresol	10.19[16]	0.49	+	++	+
3,5-Dimethylphenol	10.23[17]	0.50	—	—	—
3,4-Dimethylphenol	10.43[17]	0.45	+	—	+
2,5-Dimethylphenol	10.46[17]	0.57	+	+	+
2,3-Dimethylphenol	10.57[17]	0.56	+	+	+
2,4-Dimethylphenol	10.63[17]	0.59	++	+	+
2,6-Dimethylphenol	10.66[17]	0.65	++	+++	+

* Literature values. Reference given for each one.
** Experimental results from this work (taken from Table I).

group, as expected. The additional steric effect in *ortho*-cresol explains the relatively great decrease of the acid strength, and a corresponding increase of the R_F value, sufficient to obtain a good separation from the *meta*- and *para*-isomers. However, the two latter compounds are not separated from each other. (A mixture is detectable by spraying with diazotized sulphanilic acid, which gives an orange spot (*meta*-) surrounded by a rose border (*para*-).)

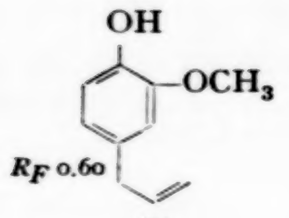
Dimethylphenols (*xylenols*) (Table III)

Of special interest in this series is the fact that from the pK_a values alone, one would expect that the difference between 2,5- and 2,3-dimethylphenol would be large enough for separation to be possible. However, the R_F values are too close and no separation is obtained. This suggests that the acidity is not the only influence on the migration on the dimethylformamide-impregnated paper.

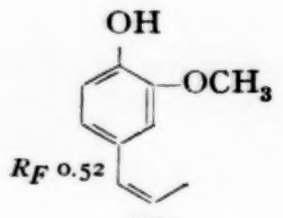
According to electronic reasoning, it would be predicted that a *para*-methyl group has an increased inductive effect ($+I$). This is confirmed by the pK_a values of 3,4- and 2,4-dimethylphenols, compared with those of the 3,5- and 2,5-; 2,3-isomers respectively. 2,4-Dimethylphenol has also a slightly higher R_F value than the isomers with the second methyl group in *meta*-position. In the case of 3,4- and 3,5-dimethylphenol, the R_F values are in inverse proportion to the pK_a values, but different enough to give separation.

Further examples (not shown in Table III) which demonstrate clearly the influence of the steric effect of an *ortho* substituent are given by *para*- and *ortho*-*tert*.-butylphenol (R_F 0.70 and 0.81 respectively). These are easily separated from each other, as are *para*- and *ortho*-phenylphenol (R_F 0.40 and 0.51 respectively).

It is remarkable and interesting to note the good separation of eugenol (I) and isoeugenol (II).



(I)



(II)

The double bond in *conjugation* with the aromatic nucleus in isoeugenol increases the polarity of the molecule, which may be predicted from the greater stability of the anion (delocalization of the charge, mesomeric effect, $+M$). The same explanation is valid for *p*-phenylphenol (R_F 0.40) and *p*-benzylphenol (R_F 0.48).

This brief discussion of some of the relationships between R_F value, acid strength and structure, based on experimental results, should aid in the interpretation of the electronic and steric effects of substituted phenols in relation to paper-chromatographic results. This may be of some help in the identification of unknown compounds.

ACKNOWLEDGEMENTS

The author wishes to thank the Directors of Firmenich & Cie. for permission to publish this work, as well as Dr. M. WINTER for his interest in the work and A. KROPF for the photographs. He gratefully acknowledges the skilful technical assistance of A. SACCARDI.

SUMMARY

A convenient two-phase solvent system for descending paper chromatography of free, mono-phenolic compounds is described. Some relationships between R_F value, acidity (pK_a) and structure are discussed.

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QUANTITATIVE PAPER CHROMATOGRAPHIC DETERMINATIONS

I. COUMARINS AND PHENOLIC ACIDS, ESPECIALLY ESCULETIN, DAPHNETIN AND FERULIC ACID

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(Received February 7th, 1961)

Phenolic acids and coumarin derivatives constitute a field of chemical and biochemical research to which increased interest has been paid in the last decades. The widespread occurrence of these compounds in nature has been reported and their possible physiological roles have been stressed. Although several efficient methods have been described for the qualitative identification of coumarins and phenolic acids in various materials, only few quantitative determinations of the compounds mentioned above have been published. Quantitative paper chromatographic methods are needed, and although it is well known that after elution from the paper, phenols can be assessed by means of the Folin-Ciocalteu reagent, too many precautions have to be taken and doubtful results are obtained due to the non-specificity of the reagent. The fact that the Folin-Ciocalteu reagent also reacts with other reducing substances is responsible for the variable blanks, and it has already been mentioned[1] that especially paper irrigated with butanol–acetic acid–water (4:1:5, v/v/v) gives such high and variable blank values. Therefore a more convenient method has been elaborated for esculetin, daphnetin and ferulic acid.

The compounds were dissolved in the minimum amount of ethanol and then further diluted with water to an appropriate concentration. 10 to 100 μg were applied with suitable constriction micropipettes on the reference line of an acetic acid washed and phosphate buffered (pH 7.4) Whatman No. 1 paper. After irrigation of the paper with *sec.*-butanol–water (4:1, v/v) the chromatogram was dried and the spots were located by their fluorescence under U.V. light. In the case of esculetin special precautions have to be taken, since it has been reported that caffeic acid and esculetin are interconvertible[2,3]. After location of the spots, rectangular fragments containing the substance under investigation, were removed from the chromatogram and eluted with water in an assembly as described by ARONOFF[4]. The eluates were collected in 10 ml volumetric flasks. A paper blank was run with each experiment.

After elution, the dihydroxycoumarins and ferulic acid were determined by means of a modified STOUGHTON–PAN method[5,6]. To the eluates were added: 1 ml

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2 N H_2SO_4, 0.1 ml $NaNO_2$ 4% (w/v), 1.5 ml concentrated ammonia and enough water to bring to volume. Boiling was omitted, since heating of the reaction mixture resulted in too high blank values. The extinction of the colour produced was measured after 15 minutes with a Beckman DU spectrophotometer in the case of esculetin and daphnetin and with a Coleman Junior spectrophotometer (round cuvettes) in the case of ferulic acid. The absorption maxima of the compounds are given in Table I.

TABLE I

Compound	*Colour of the reaction mixture*	*Absorption max. mμ*
Esculetin	faint yellow	430
Daphnetin	faint brown-violet	350
Ferulic acid	yellow	430

Fig. 1 shows the standard curves. It should be noted that the standard curves obtained *in vitro* and after chromatography were identical. The method is sensitive enough to determine micro amounts of the three compounds.

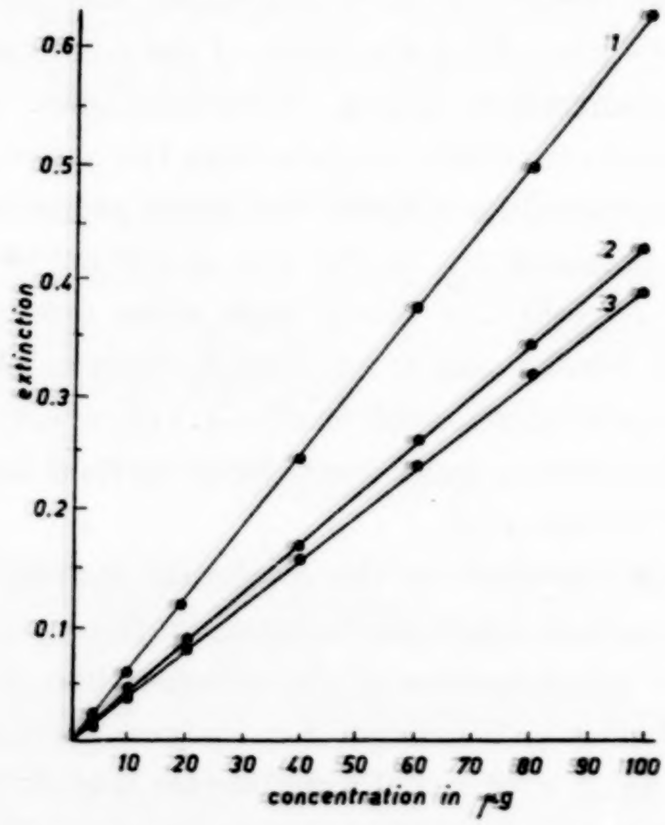

Fig. 1. Standard curves for esculetin (1), ferulic acid (2) and daphnetin (3).

o-Coumaric acid, umbelliferone, vanillic acid and *p*-hydroxybenzoic acid gave no colour under the described conditions.

SUMMARY

A method for the quantitative paper chromatographic determination of esculetin, daphnetin and ferulic acid is described. The compounds were chromatographed on phosphate buffered Whatman No. 1 paper (pH 7.4) with *sec.*-butanol–water (4:1, v/v).

After elution of the spots, the coumarins and the phenolic acid were determined by means of a modified STOUGHTON-PAN method. Standard curves obtained *in vitro* and after elution of the spots from the paper were identical.

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QUANTITATIVE PAPER CHROMATOGRAPHIC DETERMINATIONS

II. PHENOLIC ACIDS, ESPECIALLY VANILLIC ACID AND *p*-HYDROXYBENZOIC ACID

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(Received February 7th, 1961)

In our note on the quantitative paper chromatographic determination of esculetin, daphnetin and ferulic acid[1], we mentioned the fact that these three compounds could easily be determined after chromatography and elution from the paper, by means of a modified STOUGHTON–PAN method[2,3]. However, as reported, several other important phenolic acids and coumarins did not give a colour reaction with the STOUGHTON–PAN reagents under the conditions that we found favourable for quantitative paper chromatographic determinations. In order also to be able to assess the non-reacting phenolic acids, *e.g.* vanillic acid and *p*-hydroxybenzoic acid, we have developed a different method in which the compounds under investigation are measured colorimetrically after reaction with the EMERSON reagent (4-aminoantipyrine).

FUJITA AND FURUYA[4,5] employed 4-aminoantipyrine for the quantitative determination of coumarin and several of its derivatives and therefore it seemed possible to us that the same reagent could be useful in the elaboration of quantitative paper chromatographic methods for phenolic acids. In fact, EMERSON[6] and EMERSON AND KELLY[7] have found that phenols, oxidized with $K_3Fe(CN)_6$ in alkaline medium, give a colour reaction with 4-aminoantipyrine (exception should be made for phenols with certain *para*-substituents[6,8]).

The following method has proved satisfactory. The phenolic acids were dissolved in the minimum amount of ethanol and then further diluted with water to an appropriate concentration. 10 to 100 μg were applied with suitable constriction micropipettes on the reference line of an acetic acid washed and phosphate buffered (pH 7.4) Whatman No. 1 paper. Test spots were applied on either side of the starting line. After irrigation of the paper with *sec.*-butanol–water (4:1, v/v), the chromatogram was dried and the side strips were cut from the chromatogram. The spots on the side strips were revealed by spraying with diazotized *p*-nitroaniline, whereupon the side strips were again placed alongside the chromatogram in their original position.

Rectangular areas, in which the actual spots would be located on the chromatogram, were removed and eluted into 10 ml volumetric flasks by capillary washing in an assembly as described by ARONOFF[9]. A paper blank was always treated in the same way. The phenolic acids were then determined by means of the EMERSON reagent. To the eluates, 0.3 ml 0.1 *N* NaOH, 0.2 ml 4-aminoantipyrine 1% and 0.1 ml $K_3Fe(CN)_6$ 5%, were added. The red reaction mixture was shaken and further diluted with distilled water to 10 ml. The optical density was measured with a Coleman Junior spectrophotometer (square cuvettes) at 510 mμ in the case of *p*-hydroxybenzoic acid and at 500 mμ for vanillic acid. The standard curves (Fig. 1) *in vitro* and those obtained after chromatography were slightly different, the latter showing an error of about 2% in comparison with the standard curves *in vitro*. Further work with this method, in order to apply it to other compounds, is still in progress.

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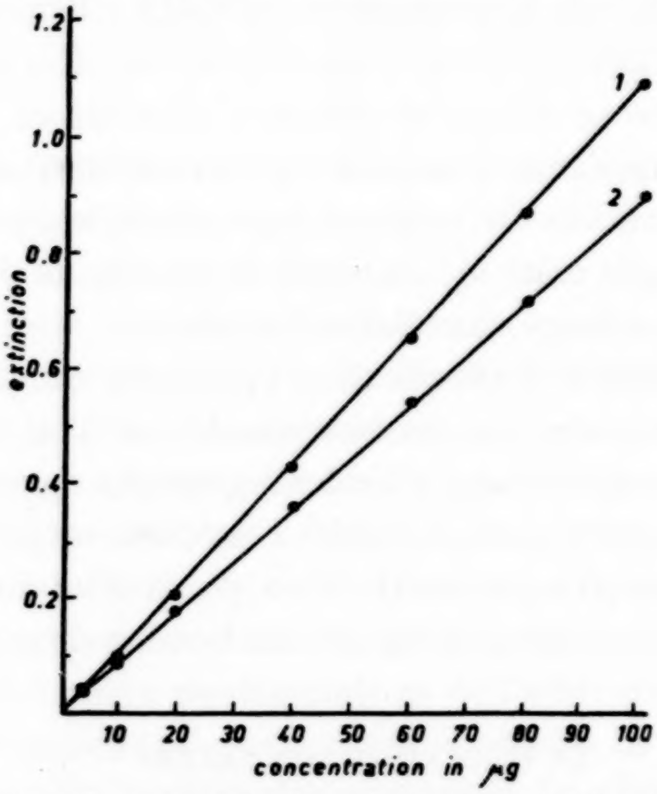

Fig. 1. Standard curves for *p*-hydroxybenzoic acid (1) and vanillic acid (2).

SUMMARY

A method for the quantitative paper chromatographic determination of vanillic acid and *p*-hydroxybenzoic acid is described. The compounds were chromatographed on phosphate buffered Whatman No. 1 paper (pH 7.4) with *sec.*-butanol–water (4:1, v/v). After elution of the spots, the phenolic acids were determined by means of the EMERSON reagent (4-aminoantipyrine). Standard curves obtained *in vitro* and after elution of the spots from the paper differed slightly (2%).

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L'ESTÉRIFICATION DES ACIDES ORGANIQUES PAR LES SOLVANTS ALCOOLIQUES

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(Reçu le 1 février 1961)

Lorsqu'un acide organique se trouve en présence d'un alcool, il est susceptible de s'unir à lui pour donner naissance à un ester; cette estérification devient plus rapide à chaud, surtout en milieu acide. Or, en physiologie végétale, où l'on étudie de plus en plus les acides organiques, la méthode classique de fixation et d'extraction comporte un séjour plus ou moins prolongé dans l'alcool bouillant.

Cependant, les possibilités d'estérification paraissent complètement méconnues, ou tout au moins sous-estimées par les biochimistes, et l'on voit très souvent des chromatogrammes ou bien des dosages d'acides organiques après passage sur colonnes de célite ou de silice qui présentent, à l'endroit précisément où se situent les esters acides, des taches ou des pics surmontés d'un point d'interrogation. Cette étude voudrait contribuer à faire disparaître un certain nombre de ces inconnus.

LA FORMATION DES ESTERS

Il n'est pas douteux que des estérifications plus ou moins abondantes se produisent au cours de la fixation ou de l'extraction du matériel végétal dans l'alcool bouillant[1]. Cette production augmente si le matériel est abandonné pendant un certain temps avant l'étude en contact avec l'alcool éthylique. Un équilibre d'estérification tend à se faire, équilibre qui se réalise plus ou moins complètement dans le vin qui vieillit.

Divers types d'esters peuvent être formés. Avec les monoacides, tels que l'acide lactique, il se forme du lactate d'éthyle qui échappe désormais à toutes les études d'acides.

Avec les diacides symétriques, tels que l'acide succinique ou l'acide tartrique, il se forme un ester acide et un ester neutre, le monosuccinate d'éthyle et le disuccinate d'éthyle.

Avec les diacides dissymétriques, tels que l'acide malique, il se forme un ester neutre et deux esters acides suivant que l'un ou l'autre des deux carboxyles est estérifié.

Avec les triacides, le nombre d'esters possibles devient plus grand: l'acide citrique, par exemple, peut former deux monoesters, deux diesters et un triester neutre, tandis que l'acide aconitique, parce que dissymétrique, peut former trois monoesters, trois diesters et un triester neutre.

Les esters se forment biologiquement par suite de l'intervention des estérases ou bien chimiquement par action de masses.

La formation d'acétate d'éthyle dans le vin qui se pique est due à l'action d'une estérase produite par le *Mycoderma aceti*. Puisque dans l'étude du matériel végétal, le premier contact avec l'alcool se fait au moment de la fixation dans ce liquide bouillant dont le but est précisément de détruire les diastases et les microbes, l'action des estérases peut être négligée.

La formation d'esters par voie chimique est régie par les lois d'équilibre, et si, comme c'est le cas dans les fixations et extractions alcooliques, la quantité d'alcool dépasse de beaucoup celle des acides organiques, l'équilibre est déplacé vers l'estérification.

Il est difficile de déterminer *a priori* quels sont, dans un milieu complexe les acides qui s'estérifient les premiers et ceux qui le font en plus grande quantité, car la vitesse d'estérification est fort mal connue pour la plupart et les études qui ont été faites sur cette vitesse l'ont été ordinairement avec des quantités équimoléculaires d'acide et d'alcool[2]. On sait cependant que dans la série aliphatique, la vitesse d'estérification, mais aussi la vitesse de saponification sont d'autant plus grandes que la chaîne carbonée est moins longue. C'est ainsi que l'ester oxalique se forme beaucoup plus vite que l'ester succinique, mais il disparaît en 3 ou 4 jours, tandis que l'ester succinique met 6 mois à se décomposer[3]. D'autre part, les esters acides sont moins stables que les esters neutres, et, dans un milieu où se trouve, par exemple, du monooxalate d'éthyle, il se formera du dioxalate et de l'acide oxalique.

Dans un milieu complexe tel que le matériel végétal, de nombreuses circonstances interviennent dont les plus importantes sont le pH, le solvant, la concentration et la température.

En principe, une fonction acide ne peut être estérifiée que si elle est libre. Or, les fonctions acides organiques sont toujours plus ou moins salifiées. L'acide citrique, par exemple, a trois fonctions acides dont le p*K* est 3.74, 4.77 et 6.4. Pour un pH de 4.77 cet acide aura une fonction libre, une fonction neutralisée et une fonction à moitié libre, à moitié neutralisée. Il se fera très facilement du monoester sur la fonction à p*K* 6.4 et un peu sur la fonction à p*K* 4.77 qui n'est qu'à moitié libre, mais sur laquelle la vitesse est plus grande; la fonction à p*K* 3.74 doit rester normalement hors de la réaction. Il est donc vraisemblable qu'il se formera relativement beaucoup de monoesters, un peu de diesters et théoriquement pas de triester. Si le pH est plus élevé et se situe aux environs de 6 ou de 7, il se formera surtout des esters avec la fonction de p*K* 6.4, mais les diesters deviendront rares.

D'après ces données, il semblerait qu'on puisse éviter toute estérification en maintenant le milieu à un pH supérieur à celui de la fonction acide qui possède le p*K* le plus élevé. Cette condition n'apporte cependant aucune certitude parce qu'interviennent le solvant, la concentration et la température.

Il nous faut tout d'abord rappeler la différence qui existe entre le p*K* et le pH:

un acide faible de pK 4 ne sera que légèrement salifié à pH 4, et pour que cette salification devienne à peu près complète, il faudra dépasser le pK d'au moins deux unités et arriver au pH 6.

De plus, n'oublions pas que la constante de dissociation est définie pour un milieu aqueux homogène. En milieu hydro-alcoolique, cette constante diminue lorsque la teneur en alcool augmente: avec 20% d'alcool, la constante diminue de 50%; le pK de chaque fonction acide augmentera donc avec le pourcentage d'alcool. Ainsi s'explique, par exemple, qu'en solution alcoolique l'acétate de potassium dont le pK est de 4.75 soit décomposé par le gaz carbonique dont le pK est beaucoup plus élevé, 6.37 et 10.39.

D'ailleurs, même dans l'eau, cette constante varie. En effet, les sels d'acides faibles neutralisés par des bases fortes forment des ions susceptibles de réagir avec les ions de l'eau:

$$AB \rightarrow A^- + B^+ + H_2O = AH + OH^- + B^+$$

Le degré d'hydrolyse du sel AB sera défini approximativement par une relation où nous introduisons un terme correctif pour tenir compte du fait que, dans le milieu où nous opérons, il convient de considérer les activités plutôt que les concentrations, car celles-ci sont inutilisables dans les calculs, par suite de la multitude des corps dissous et de la présence de l'alcool:

$$\beta = \sqrt{\frac{K_{H_2O}}{K_A}} \times \frac{1}{c}\sqrt{\frac{y_{A^-}}{y_{OH^-}}}$$

De cette formule il découle que le degré d'hydrolyse étant inversement proportionnel à la concentration du sel dissous c, plus celle-ci sera faible par rapport au solvant, plus l'hydrolyse sera importante. Or, les quantités d'acides organiques sont vraiment très faibles par rapport à la masse du solvant employé pour la fixation et l'extraction.

Enfin, nous voyons à quel point peut influer la température puisque le degré d'hydrolyse varie dans le même sens que K_{H_2O}. Or, le K de l'eau qui à 20° est de $0.68 \cdot 10^{14}$ passe pour 100° à $58.2 \cdot 10^{14}$ soit à une valeur près de cent fois supérieure; la dissociation des acides variera donc dans le même sens et dans les mêmes proportions.

Ainsi donc est-il difficile de prévoir avec exactitude la quantité d'ester qui se formera à partir de tel acide organique lors de la fixation initiale ou du séjour en milieu alcoolique, mais il est sûr qu'il s'en formera une certaine quantité. Il peut même se former des esters neutres avec des polyacides dont une fonction acide paraissait tout de même bien protégée par le fait que son pK était bien inférieur au pH de la solution.

Nous allons surtout parler des esters acides parce qu'on les trouve en étudiant les acides: ils sont retenus par les mêmes résines échangeuses d'ions, révélés avec les mêmes révélateurs et dosés de la même manière. Les esters des monoacides, ainsi que les esters neutres nous échappent totalement dans ces conditions parce qu'ils ne réagissent plus comme les acides.

LE COMPORTEMENT DES ESTERS EN CHROMATOGRAPHIE

1. *En chromatographie sur papier*

Les esters se séparent fort bien des acides comme on pouvait le prévoir, puisque disparaît par estérification une de ces fonctions dont la présence diminue considérablement le R_F. Les diesters se séparent eux aussi des monoesters, et nous voyons, par exemple, l'acide citrique passer de la zone des triacides dans celle des diacides avec les monoesters, et dans celle des monoacides avec les diesters. En revanche, les différents monoesters et les différents diesters du même acide ne se séparent pas l'un de l'autre, dans les conditions où nous opérons.

Dans le Tableau I sont présentés avec notre système de solvants, éthanol–ammoniaque et butanol–formique, sur papier Arches 302 les R_F, ou plus exactement les R_G (acide glycolique) de quelques acides et ceux de leurs esters éthyliques, avec les valeurs de l'acide lactique comme référence.

TABLEAU I

	R_G	
	Ethanol–ammoniaque	*Butanol–ac. formique*
Ac. lactique	145	120
Ac. aconitique[1]	20	135
monoaconitate	75	155
diaconitate	230	165
Ac. citrique	15	75
monocitrate	70	125
dicitrate	220	150
Ac. malique	40	90
monomalate	180	135
Ac. tartrique	30	50
monotartrate	130	105
Ac. malonique	30	100
monomalonate	170	145
Ac. succinique	50	125
monosuccinate	200	150
Ac. maléique	45	105
monomaléate	100	140

Si, considérant ces valeurs, nous représentons le R_G des esters en milieu acide en fonction du R_G des acides dont ils proviennent, nous constatons que les points représentatifs se distribuent en courbe régulière qui paraît avoir la forme d'une fraction de branche d'hyperbole (Fig. 1). Il semble donc exister une relation simple entre ces valeurs, et ceci nous incite à rechercher si l'estérification d'un acide se traduit par une variation régulière du R_M pouvant nous permettre de trouver une loi d'additivité analogue à celle que [illegible][2] indique pour les acides organiques :

$$R_M = \log \frac{R_F}{1 - R_F}$$

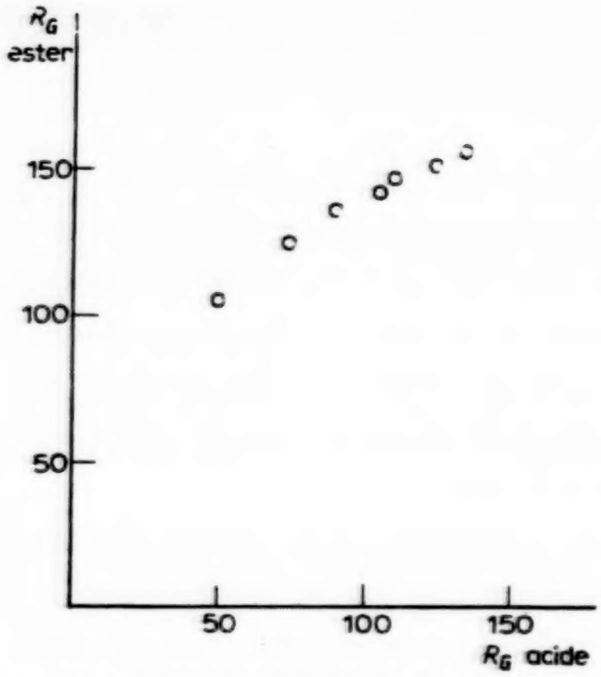

Fig. 1. R_G des esters en milieu acide en fonction du R_G des acides dont ils proviennent.

Puisque nous obtenons les R_G en prenant comme égale à 100 la valeur du R_F de l'acide glycolique qui est en réalité 0.60, nous pouvons calculer les R_M à partir des R_G en remplaçant 1 par 167, soit:

$$\frac{100}{60} \times 100, \text{ et nous avons } R_M = \log \frac{R_G}{167 - R_G}$$

Dans les conditions où nous opérons, voici, suivant cette relation, quelques constantes de groupe avec le solvant acide: + 0.82 pour la constante de base; + 0.16 pour un atome de carbone; — 0.50 pour un carboxyle, etc. Le changement apporté par une estérification doit pouvoir être représenté par une constante que nous trouverons en comparant les valeurs des R_M des acides avec celles de leurs esters éthyliques (Tableau II).

TABLEAU II

	R_M d'acide	R_M d'ester	ΔR_M
Ac aconitique	0.63	1.12	0.49
Ac. citrique	—0.09	0.48	0.57
Monocitrate d'éthyle	0.48	0.95	0.47
Ac. malique	0.07	0.53	0.46
Ac. tartrique	—0.37	0.23	0.60
Ac. malonique	0.29	0.83	0.54
Ac. succinique	0.48	0.95	0.47
Ac. maléique	0.23	0.72	0.49

Les ΔR_M indiqués dans la dernière colonne oscillent autour d'une valeur moyenne. Cette valeur est déterminée graphiquement en représentant les R_M des esters en fonction des R_M des acides (Fig. 2): les points se distribuent approximativement sur une droite qui coupe l'axe des ordonnées en un point, 0.55, que nous avons choisi comme valeur pratique de calcul $\Delta R_M = 0.55$.

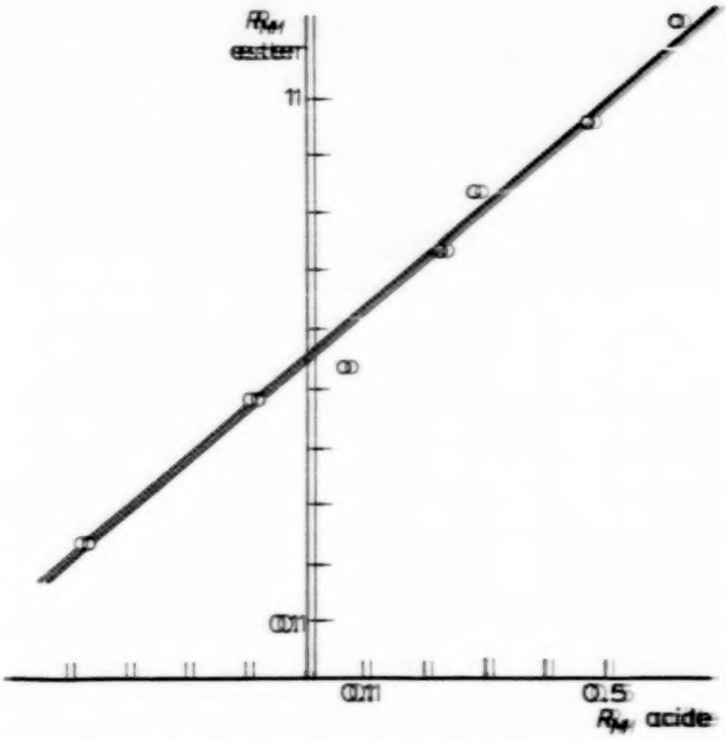

Fig. 2. R_M des esters en fonction des R_M des acides.

Nous pouvons dès lors calculer, à partir du R_F des acides, celui de leurs esters éthyliques. Le Tableau III montre une concordance stricte entre les valeurs calculées et les valeurs mesurées expérimentalement.

TABLEAU III

	Valeur du R_F		ΔR_F
	mesurée	*calculée*	
Monoaconitate	0.93	0.93	0
Monocitrate	0.75	0.742	—0.008
Dicitrate	0.90	0.91	+0.01
Malate	0.81	0.80	—0.01
Tartrate	0.63	0.60	—0.03
Malonate	0.87	0.87	0
Succinate	0.90	0.90	0
Maléate	0.84	0.85	+0.01

Il existe donc une relation simple entre les R_G des acides et celui des esters et nous pouvons écrire:

$$R_{M\ \text{ester}} = a\, R_{M\ \text{acide}} + b$$

qui devient avec $Y = R_{G\ \text{ester}}$ et $X = R_{G\ \text{acide}}$ l'équation:

$$\log \frac{Y}{167 - X} = a \log \frac{X}{167 - X} + b$$

La résolution de cette équation montre que la loi de variation des $R_{G\ \textbf{ester}}$ en fonction des $R_{G\ \textbf{acide}}$ est de la forme $Y = \dfrac{AX}{BX + C}$ ou plutôt $Y = \dfrac{AX + D}{BX + C}$ pour tenir compte du fait qu'expérimentalement la courbe ne passe pas par l'origine.

Nous arrivons ainsi à l'équation $Y = \dfrac{225\,X + 625}{X + 65}$ qui nous permet de calculer avec une très bonne approximation le R_G des esters à partir du R_G des acides (Fig. 3) (Tableau IV).

TABLEAU IV

	R_G des acides	R_G des esters		ΔR_G
		mesurés	*calculés*	
Ac. aconitique	135	155	155	0
Ac. citrique	75	125	125	0
Ac. malique	90	135	134.6	—0.4
Ac. tartrique	50	105	103.2	—1.8
Ac. malonique	110	145	145	0
Ac. succinique	125	150	150	0
Ac. maléique	105	140	139	—1

Ces mêmes calculs pourraient être faits avec les R_G du solvant alcalin, bien que l'additivité soit un peu moins bonne.

Quoiqu'il en soit, la carte chromatographique de ces esters montre qu'ils vont se grouper surtout dans la région de l'acide lactique (Fig. 4).

La révélation de ces taches est moins nette que celle des acides, surtout avec les révélateurs basés sur l'acidité. Ces esters gardent cependant quelque chose de la réaction des acides dont ils sont issus et le monotartrate d'éthyle, par exemple, présente avec le nitrate d'argent une évolution assez nette vers le châtain et le marron.

2. *En chromatographie sur colonne*

Lorsque, pour doser les acides organiques, on les fait passer sur colonne de silice ou de célite, les esters se séparent fort bien des acides et les monoesters des diesters. Cependant, les esters sortent tous avec le premier solvant, et leur ordre de sortie est si rapproché l'un de l'autre qu'il faut, pendant le passage de ce premier solvant, prendre les fractions les plus petites possible, si l'on veut essayer de les séparer.

Pour les séparer un peu mieux, on peut, avant le premier solvant, en utiliser un autre qui soit moitié moins riche en butanol (4% au lieu de 8%): ce solvant fait sortir les esters monoacides. Certains, comme le dicitrate d'éthyle, peuvent même sortir sans butanol avec le seul chloroforme.

Mais, considérons seulement les solvants classiques. Il est assez difficile de donner des précisions sur les résultats obtenus par suite de la variété des techniques employées. Voici cependant l'ordre et la place de sortie de quelques esters dans la méthode que nous utilisons et qui n'est guère autre chose qu'une adaptation de celle de BOVÉ ET RAVEUX[6] à de plus petites quantités. Avec une colonne de 0.65 cm^2 de section contenant 3 g de célite et un premier solvant de 42 ml dont 8% de butanol et 92% de chloroforme, l'acide fumarique sort au 35ème ml, le monocitrate au 33ème et le dicitrate au 4ème, le monomalate au 14ème, le tartrate au 20ème, etc.

Les esters sortent donc dans le premier solvant quand ils ne sont pas saponifiés,

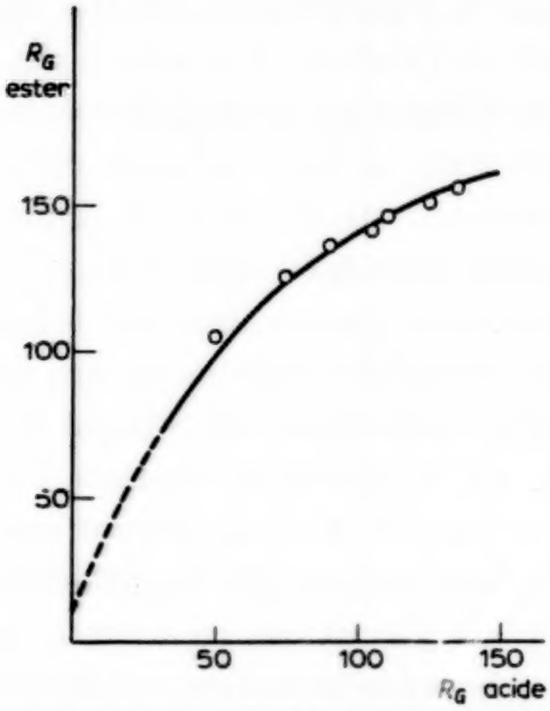

Fig. 3. R_G des esters calculé à partir du R_G des acides.

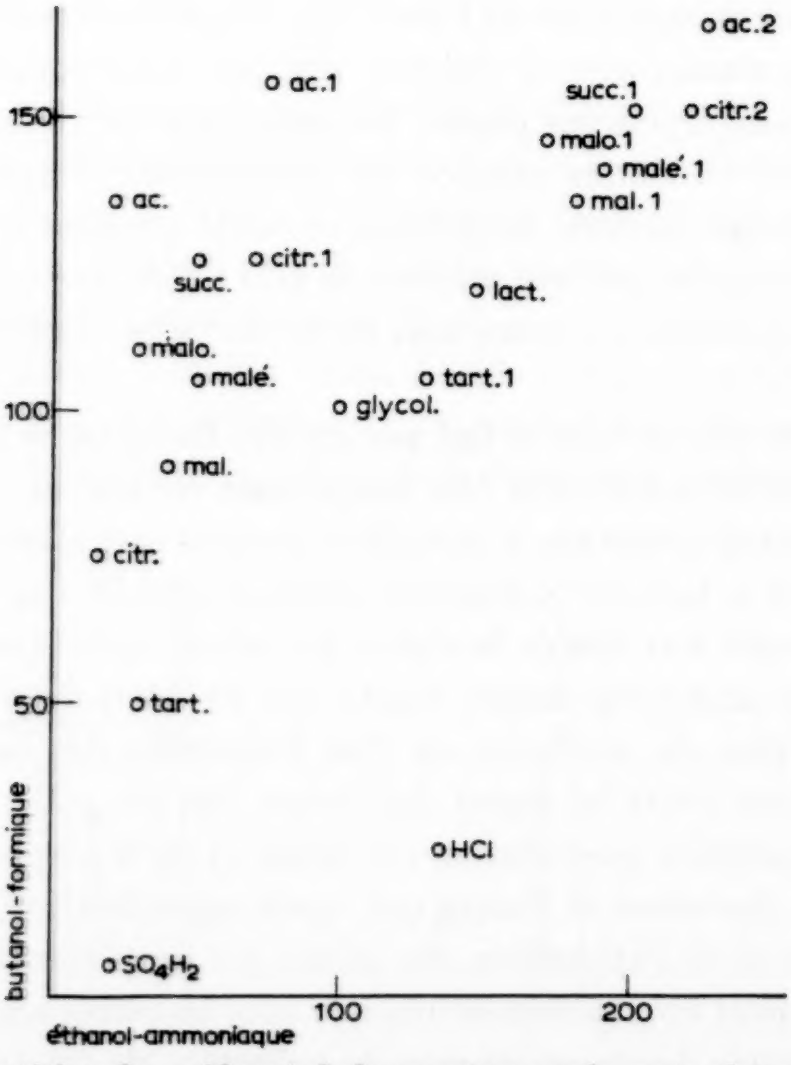

Fig. 4. Répartition des acides et de leurs esters sur la carte chromatographique.

mais il existe une possibilité que l'on ne saurait exclure, à savoir la saponification des esters sur la colonne.

Pour l'acide oxalique dont la saponification est très rapide, elle se fait, si elle n'est déjà faite, avant le départ de l'élution: il ne nous a pas été possible d'obtenir la moindre trace de mono-oxalate d'éthyle au sortir de la colonne. Pour les autres acides, il est possible qu'une saponification se fasse entre le début et la fin de l'élution. Supposons qu'il s'agit d'un monocitrate d'éthyle: il commence à se déplacer à la vitesse de l'ester, mais la saponification intervient et la vitesse devient alors celle de l'acide. Ces portions d'acide citrique réparties ainsi tout le long de la colonne risquent de se présenter pour le dosage comme des traînées ou bien de petits pics survenant à des endroits inattendus. En effet l'ester était emporté par le premier solvant, mais ce solvant, ainsi que le suivant, est incapable de déplacer l'acide citrique qui restera donc sur place dans la colonne jusqu'à ce qu'un solvant assez puissant balaie toutes ces portions d'acide pour en former un petit pic supplémentaire qui marque l'endroit où serait sorti l'acide citrique s'il n'avait pas eu toute la longueur de la colonne à parcourir. Certains petits pics, de faible importance d'ailleurs, paraissent avoir cette origine.

L'IDENTIFICATION DES ESTERS

De nombreuses réactions sont possibles pour identifier les esters, mais elles ne nous ont guère donné satisfaction par suite de la faible quantité d'acide ordinairement estérifiée. Nous n'en citerons qu'une, celle de FEIGL[7] qui transforme les esters en acides hydroxamiques pour les révéler avec le chlorure ferrique. Voici comment nous l'avons appliquée à la chromatographie sur papier. Le papier porteur de la tache d'ester est imbibé d'une solution alcoolique saturée de chlorhydrate d'hydroxylamine, puis d'une solution alcoolique saturée de potasse et porté pendant 5 min à l'étuve à 105–110°. Après acidification par une solution de HCl 0.5 *N*, une solution de chlorure ferrique à 1 % fait apparaître les esters sous forme de taches violettes plus ou moins nettes.

L'identification la plus précise se fait par les R_F. Toute tache apparaissant dans la région où se situent les esters doit être soupçonnée comme un ester possible: on refait le même chromatogramme après saponification pour voir si ces taches persistent. La saponification peut se faire au bain-marie bouillant avec HCl ou SO_4H_2 (2 ou 3 *N*) dont les taches se situent fort loin de la région des esters: un tel traitement ne paraît pas affecter les autres acides organiques, tandis que les esters disparaissent.

Pour arriver à plus de certitude, on élue l'ensemble des taches soupçonnées être des esters, ou bien toute la région des esters. On en refait deux chromatogrammes, l'un qui montrera que l'élution est bonne et qu'il n'apparaît pas de tache en dehors de la zone des esters et l'autre qui, après saponification, témoignera de la disparition des esters et de l'apparition des acides qui leur avaient donné naissance. Une telle expérience peut être facilement réalisée avec les esters acides du vin (Fig. 5) ou même avec l'ensemble des premiers pics du collecteur de fractions après passage sur célite ou sur silice.

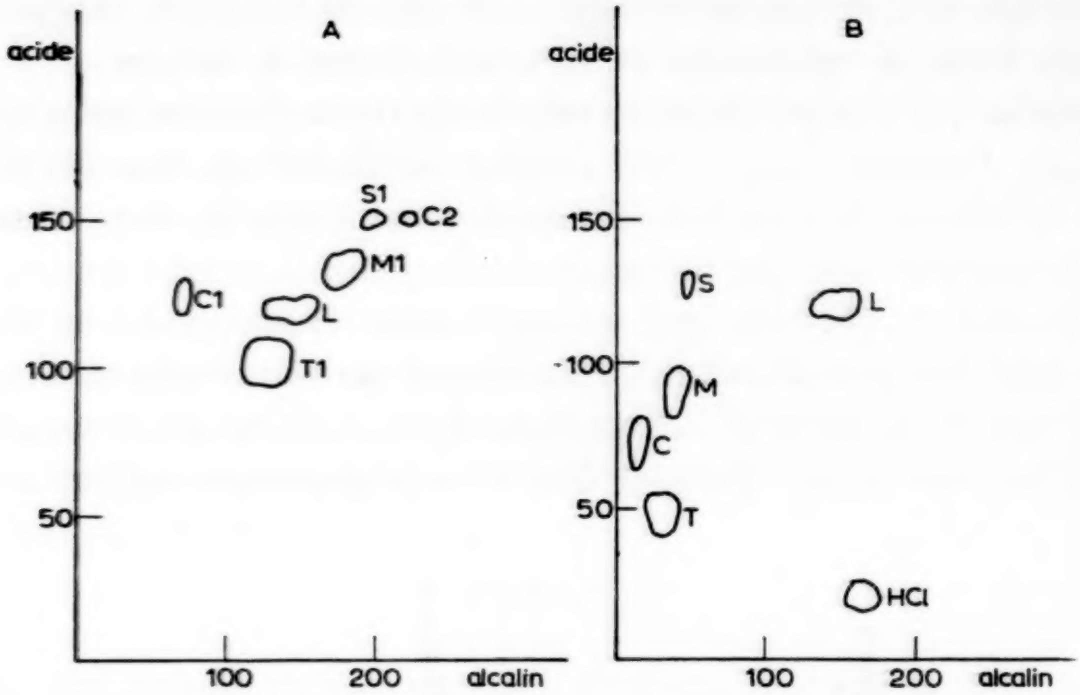

Fig. 5. Carte des esters acides du vin: (A) avant hydrolyse; (B) après hydrolyse. C1 = monocitrate; C2 = dicitrate; L = lactique; M1 = monomalate; S1 = monosuccinate; T1 = monotartrate.

LE DOSAGE

Le dosage des esters acides ne soulève pas de difficultés spéciales, si ce n'est que la séparation de ces esters est assez délicate sur colonne, et que les fractions doivent, comme nous l'avons dit, être très petites si l'on ne veut pas se contenter d'un dosage global.

Prenons par exemple un dosage de l'acide citrique et de ses esters. Le premier pic qui se situe au 4ème ml de solvant représente le dicitrate d'éthyle, le second au 33ème représente le monocitrate et le troisième, beaucoup plus loin, l'acide citrique (Fig. 6). Le dosage du premier s'est fait avec 10.7 ml de soude 0.01 *N*, celui du deuxième avec 60.8 ml et celui du troisième avec 78 ml.

Si nous considérons l'acidité dosée, l'acide citrique représente 52 % de cet ensemble, le monoester 40.8 et le diester 7.15 %, mais si nous évaluons en acide citrique, le diester sera trois fois plus abondant et le monoester sera multiplié par 1.5. Nous

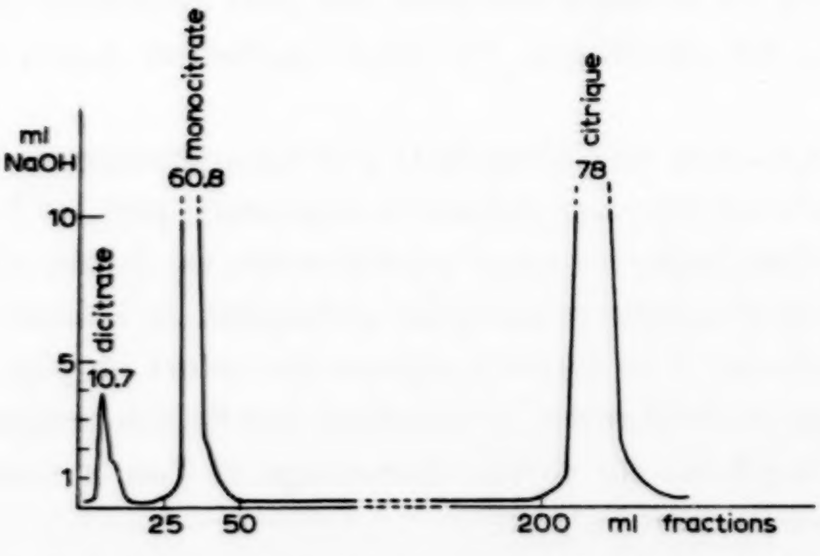

Fig. 6. Dosage de l'acide citrique estérifié.

constatons ainsi que 39% de l'acide citrique n'ont pas été estérifiés, tandis que 45·5 % sont passés sous forme de monoester et 16 % sous forme de diester.

Nous ne tenons pas compte dans ces calculs du triester neutre qui n'apparaît pas dans ces dosages. Pourtant, dans cette solution où l'acide citrique est seul présent dans le milieu alcoolique où s'est faite artificiellement l'estérification, nous pourrions arriver à une évaluation assez approximative de la quantité d'ester neutre, mais dans les cas concrets cette évaluation devient impossible. Hâtons-nous de dire que ces esters neutres sont très peu abondants, étant donné surtout le pH relativement élevé dans lequel se fait la fixation et l'extraction. Les esters neutres, beaucoup moins abondants que les esters acides peuvent donc être pratiquement négligés.

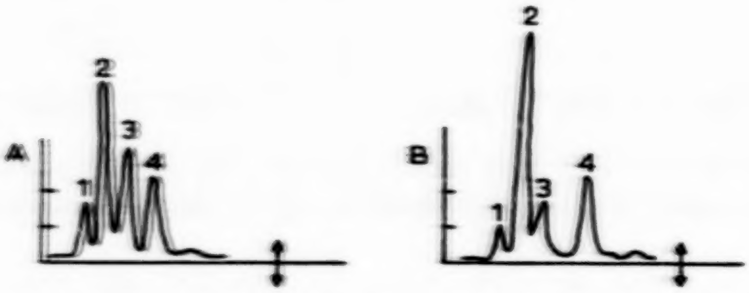

Fig. 7. Graphique du dosage des pics du premier éluant. (A) Avec autant de citrique que de malique. (B) Avec cinq fois plus de malique que de citrique. 1 = dicitrate; 2 = malate; 3 = monocitrate; 4 = fumarique.

Dans le cas le plus favorable, les pics des différents esters ont été séparés et identifiés et le dosage est simple, mais il arrive assez souvent que cette séparation ne puisse se faire avec netteté. La question se pose alors de savoir si nous pouvons, dans ce dosage global, étant donné l'ensemble des acides organiques présents, évaluer avec assez de probabilité la part qui revient à chacun.

Il est bien évident que l'oxalate est trop instable pour se trouver parmi les esters, mais il est vraisemblable que l'ensemble des esters des autres acides s'y trouve. En première approximation, on peut admettre une égale proportion d'esters pour tous et chacun des acides. Si, par exemple, le total des esters représente un cinquantième de l'ensemble des acides, il conviendra d'augmenter d'un cinquantième le dosage de chacun des acides, car, et cette probabilité n'est pas contredite par les faits, il est vraisemblable que si l'acide malique est deux fois plus abondant que l'acide citrique et 50 fois plus que l'acide succinique, les esters garderont à peu près entre eux la même proportion.

De telles évaluations sont assez loin de la précision absolue mais elles permettent de corriger les valeurs trouvées, aux erreurs d'expérience près, et l'on peut, quand le dosage individuel est impossible, et que l'estérification est faible, s'en contenter.

En conclusion, il faut admettre qu'après utilisation de l'alcool éthylique comme fixateur ou comme solvant, il se forme toujours des esters en plus ou moins grande abondance. Ils atteignent facilement le centième des acides présents, mais peuvent atteindre jusqu'au vingtième ou même davantage si l'on a conservé le matériel pendant un certain temps dans l'alcool.

Ces estérifications posent le problème de l'utilisation de l'alcool comme fixateur et comme solvant lorsqu'on étudie les acides organiques. Nous pensons qu'il est difficile de se passer d'un liquide tellement pratique comme fixateur, comme solvant et comme conservateur. Si l'on connaît les corrections à faire et qu'on en tienne compte, les erreurs deviennent négligeables.

RÉSUMÉ

En présence d'alcool comme fixateur et comme solvant du matériel végétal, il se forme toujours des esters à partir des acides organiques. Cette estérification est facilitée par la température élevée, la faible concentration des acides, etc. Il est facile de repérer ces esters sur les chromatogrammes et même de les doser car ils se séparent fort bien des acides dont ils sont issus.

SUMMARY

If alcohol is used as solvent for vegetable material or for purposes of fixation, the organic acids present in the material always form esters. This esterification is facilitated by elevated temperatures and the low concentration of the acids, etc. It is easy to locate these esters on chromatograms and even to determine them, because they are widely separated from the acids from which they have been formed.

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PAPER CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATION AND IDENTIFICATION OF SOME NATURALLY OCCURRING TETRONIC ACIDS

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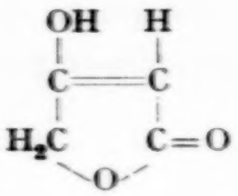
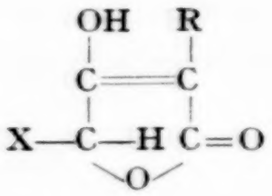
(Received February 15th, 1961)

A number of moulds are known to produce substances related to tetronic acid (I) when grown on certain defined liquid media[1–5]. These acids have the general formula shown below (II).



(I)



(II)

In this account the behaviour of five such tetronic acids is recorded. Table I shows the common names of these acids with their corresponding substituent groups.

TABLE I

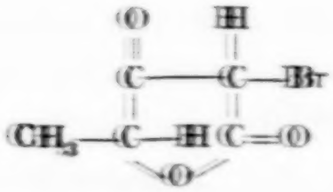
Common name	*Group X*	*Group R*
Carolic acid (hydrated form)	$-CH_3$	$-CO\cdot CH_2\cdot CH_2\cdot CH_2OH$
Carlic acid (hydrated form)	$-CH_2COOH$	$-CO\cdot CH_2\cdot CH_2\cdot CH_2OH$
Carlosic acid	$-CH_2COOH$	$-CO\cdot CH_2\cdot CH_2\cdot CH_3$
Terrestric acid (hydrated form)	$-CH_3$	$-CO\cdot CH_2\cdot CH_2\cdot CHOH\cdot C_2H_5$
Viridicatic acid	$-CH_2COOH$	$-CO\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_3$

The first three of these acids have been isolated from *Penicillium charlesii* G. Smith[1]; terrestric acid has been isolated from *Penicillium terrestre* Jensen[3]; whilst terrestric and viridicatic acids have been obtained from *Penicillium viridicatum* Westling[5]. HAYNES AND PLIMMER[6] have recently discussed the properties and, in particular, the strongly acidic nature of some tetronic acids.

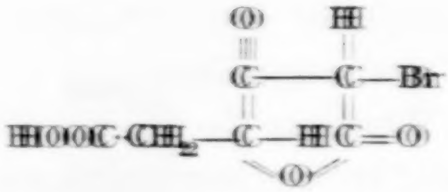
DETECTION OF TETRONIC ACIDS

In the course of investigations into the metabolism of *Penicillium charlesii* G. Smith, the need arose for a sensitive and specific method for the separation and detection of tetronic acids. REIO[7] included the tetronic acids in his chromatographic survey of mould metabolites, and HAYNES, PLIMMER AND STANNERS[8] have described the chromatographic behaviour of carolinic acid (formula III, R = $COCH_2CH_2COOH$; X = CH_3). At the time when the present work was started, however, only the latter report had appeared in the literature. REIO[7] detected the acids on paper chromatograms using a bromophenol blue indicator spray. He also made use of the reaction between aqueous $FeCl_3$ and certain tetronic acids[1,3-5]. Present work confirmed his results that spots containing 25 to 50 μg of a tetronic acid do not show up strongly with the bromophenol blue spray. In order to detect tetronic acids with an aqueous $FeCl_3$ spray, similar amounts are necessary but again the reaction is weak and the yellow spots are often difficult to see.

The detection method used in the present work is based on the reaction between the tetronic acids and bromine as described by CLUTTERBUCK *et al.*[9,10]. In 50 % glacial acetic acid carolic and carolinic acids gave mainly α-bromo-γ-methyl-tetronic acid (III), whilst with carlosic and carlic acids an analogous reaction occurred with production of α-bromo-γ-carboxymethyl-tetronic acid (IV).



(III)



(IV)

The tetronic acids are converted to their corresponding bromo derivatives on paper chromatograms by spraying with bromine in acetic acid solution. The bromo compounds are then revealed by spraying with a solution of starch–KI. Similar spray reagents have been used for the detection of peptides[11] and other nitrogenous compounds[12].

Spray reagent and procedure

A solution of 0.1 ml Br_2 in 100 ml 50% glacial acetic acid is sprayed on to the dry chromatograms; a light spray suffices. When the paper is again completely dry, after 10–20 min in air, a second spray of 1 % (w/v) soluble starch solution containing 2 % (w/v) KI is applied. The tetronic acids show up immediately as blue or brown spots on a white background. The spots are marked as soon as the paper is dry as they tend to fade, particularly after using an alkaline chromatography solvent. The reagent easily detects 5 μg of each of the acids tested, after chromatography using any of the solvents discussed below. Following chromatography using alkaline or neutral solvents the sensitivity of the reagent may be increased by spraying lightly with N/10 HCl after the starch–KI spray.

Specificity of the reagent

The specificity of the spray reagent was tested against some common organic acids on

Whatman No. 1 paper. The paper was developed with the Br_2/starch–KI reagent as described above. The following acids, at levels as high as 200 μg, gave no colour with the reagent: citric, isocitric, *cis*-aconitic, malic, succinic, fumaric, tartaric, and itaconic. A faint blue colour was given by 100 μg of oxoglutaric acid. With 100 μg, pyruvic, oxaloacetic and malonic acids gave strong blue colours. The reagent apparently detects acids which are capable of enolising. (Malonic acid may be regarded as an enol[13].)

PAPER CHROMATOGRAPHY

A series of buffered solvent systems were used because the pH could be adjusted in order to effect particular separations. These are prepared as follows: *n*-butanol is saturated by shaking up with *M*/10 phosphate buffer of definite pH. Whatman No. 1 papers are thoroughly soaked by pulling the papers through a trough of the same buffer. The papers are then hung to dry so that the excess buffer drips off the paper in the same direction as the eventual chromatography solvent flow. The papers are allowed to dry in air for 6 h and are then irrigated with the buffer-saturated butanol. Buffer systems of pH 3, 5 and 7 (designated A, B and C respectively) gave the best separations of the available acids. Chromatograms were also irrigated with the top layer of a *n*-butanol–water–pyridine (5:4:1, by vol.) mixture (D). All chromatograms were run for 16 h on Whatman No. 1 paper using the descending flow method. Chromatograms were irrigated in the complete absence of any "aqueous phase" since the best separations were achieved under these conditions with all the solvents which were used. Some typical R_F values are listed in Table II. These values were not found to be strictly reproducible but the relative mobilities of the acids were constant for a particular solvent system.

TABLE II

R_F VALUES OF TETRONIC ACIDS

Acid	$R_F \times 100$			
	A	*B*	*C*	*D*
Carolic	26	27	25	38
Carlic	8	6	0	—
Carlosic	26	22	2	9
Terrestric	73	58	52	60
Viridicatic	47	43	5	24

HIGH VOLTAGE PAPER ELECTROPHORESIS

The apparatus used was a modification of that described by GROSS[14]. Tap water circulating through the precision-ground aluminium plates provides the cooling system. Wicks of folded Whatman 3 MM strips complete the connection between the electrodes and the electrophoretic strip. The wicks are wrapped in cellophane (British Cellophane Ltd., grade 300 P.T.) in order to minimise the inflow of buffer from the wicks to the strip which otherwise may cause movement and distortion of spots. The electrophoretic strips (4 1/2 × 22 1/4 in. Whatman 3 MM papers) are dipped through a trough of buffer and then passed through a domestic clothes wringer to remove excess buffer. The mixture to be separated is spotted on to the wet strip, or preferably, applied as a thin streak using a device similar to that described by OSBORNE AND BAWDEN[15]. Of the buffers tested, a pyridine–acetic acid–water mixture (10:0.4:190, by vol.) of

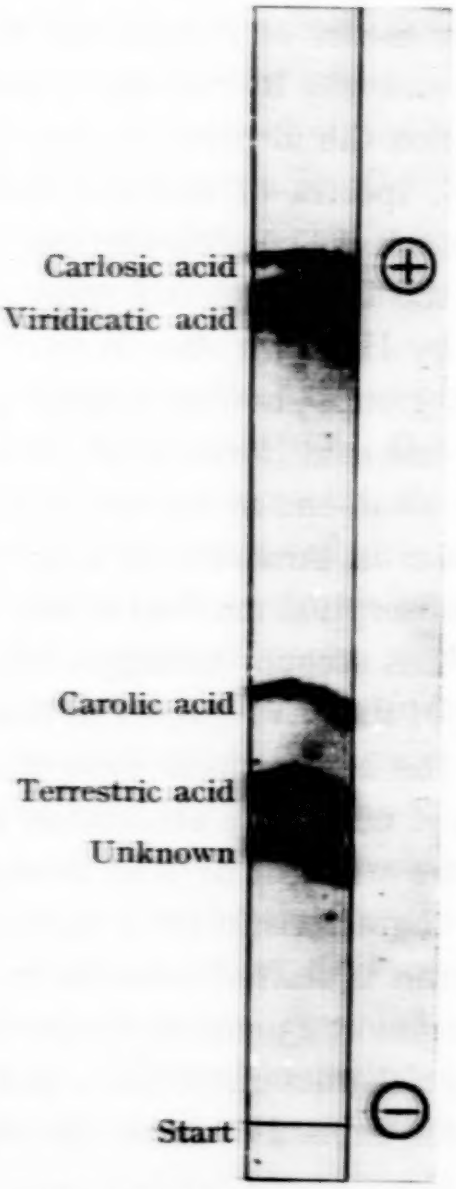


Fig. 1. Electrophoresis of a mixture containing approximately 20 μg each of terrestric, carolic, viridicatic and carlosic acids, and an unknown tetronic acid, applied as a streak 6 in. from cathode end. Conditions: 190 V/cm; 1 mA/cm; 35 min; Whatman No. 3 MM 4 1/2 × 22 1/4 in. strip; pyridine–acetic acid–water buffer (10:0.4:190, by vol.), pH 6.5. Spraying reagents: 0.1 ml Br_2 in 100 ml 50% acetic acid; 1% starch solution containing 2% KI.

pH 6.5 gave the best separation of the available acids. Fig. 1 shows a typical separation.

In Table III are listed some electrophoretic mobilities. These values are only

TABLE III

ELECTROPHORETIC MOBILITIES OF TETRONIC ACIDS

Acid	*Mobility* $cm^2/V \cdot sec \times 10^5$
Terrestric	4.0
Carolic	4.8
Viridicatic	7.4
Carlosic	8.0

approximate since the actual voltage across the paper strips is less than the applied voltage and no correction was made for endosmotic flow. Nevertheless under constant conditions the mobilities are strictly reproducible.

IDENTIFICATION OF TETRONIC ACIDS

Acids appearing in the culture media of *Penicillium charlesii* G. Smith have been tentatively identified in the usual way by running "markers" on chromatograms or electrophoretic strips. In addition the identity of some tetronic acids has been confirmed by measuring the U.V. spectra of material eluted from chromatograms or electrophoretic strips. The spectra of "possible tetronic acids", *i.e.* substances which give a positive reaction with the Br_2-starch-KI spray, may be similarly examined.

From the data recorded by HERBERT AND HIRST[16] it is clear that the tetronic acids can be divided into two groups showing characteristic U.V. spectra. The first group, typified by α-ethyltetronic acid (formula II; $R = C_2H_5$; $X = H$) show absorption maxima at 265 mμ in alkali and at 230 mμ in acid media. The second group, which consists of acids similar in structure to α-acetyltetronic acid (formula II; $R = CO\cdot CH_3$; $X = H$) have absorption maxima at 265 and 230 mμ in both acid and alkali. The acids described in this account belong to the second group.

A careful re-examination of the U.V. spectra of the available acids revealed important differences between the acids which have not been reported previously. Thus, it was found that in acid media the absorption maximum at 230 mμ can be completely suppressed, and the strength of acid necessary to completely suppress this maximum at 230 mμ is characteristic for a particular tetronic acid. Also the absorption maximum at 265 mμ is shifted towards the longer wavelengths in acid media so that when the maximum at 230 mμ is completely suppressed a single maximum at 274 mμ remains. These characteristic shifts in U.V. spectra are useful in the identification of tetronic acids. Table IV shows the strength of HCl required to suppress the various maxima at 230 mμ. (The maximum at 230 mμ is considered to be suppressed completely when the spectrum shows no obvious maximum at 230 mμ and when the optical density reading at 230 mμ is less than that at 224 mμ. With the instrument available (Unicam S.P. 500 spectrophotometer) it is not possible to decide

TABLE IV

CONCENTRATION OF HCl REQUIRED TO SUPPRESS ABSORPTION MAXIMUM AT 230 mμ

Acid	*HCl conc. N*
Carolic	0.1
Carlic	0.05
Carlosic	2.0
Terrestric	0.005
Viridicatic	2.0

whether the maximum at 230 mμ is removed or merely shifted to a wavelength below 220 mμ.)

A PROCEDURE FOR THE PARTIAL CHARACTERISATION OF NEW TETRONIC ACIDS

All the tetronic acids so far isolated from fungal sources have either a methyl or a carboxymethyl group attached to the γ-carbon atom of the ring (dehydrocarolic acid[4] is an exception, having a methylene group instead). After bromination in acetic acid solution these tetronic acids yield either α-bromo-γ-methyl-tetronic acid (III) or α-bromo-γ-carboxymethyl-tetronic acid (IV). These α-bromo derivatives are easily separable by paper chromatography so that it is possible to characterise a new tetronic acid according to the type of α-bromo derivative which it yields on bromination.

The following experiment illustrates the type of procedure that has been applied to an unknown compound. The compound ($C_9H_{10}O_5$), which gave the two-banded U.V. spectrum characteristic of some tetronic acids, was isolated from culture filtrates of *Penicillium charlesii* G. Smith. This compound gave an orange colour with aqueous $FeCl_3$ solution which is a typical reaction of tetronic acids[1,3–5] and also gave a positive reaction with the Br_2/starch–KI reagent on paper. Spots containing 50 μg of carolic, carlosic, terrestric and viridicatic acids and the unknown substance were applied along the starting line of a chromatogram (Whatman No. 1). The area containing these spots was lightly sprayed with the Br_2 in 50% glacial acetic acid reagent in order to convert the tetronic acids to their corresponding α-bromo derivatives. After drying in the fume cupboard for 2–6 h the chromatogram was irrigated for 16 h by the descending flow method using the *n*-butanol–water–pyridine system described earlier. The separated bromo derivatives were revealed by spraying the dry chromatogram with the starch–KI reagent, followed immediately by a light spray with *N*/10 HCl which increases the sensitivity of the reagent. From the results set out in Table V it seems probable that the "unknown tetronic acid" contains a γ-methyl-tetronic acid ring structure like that of carolic and terrestric acids.

TABLE V

R_F VALUES OF TETRONIC ACID BROMO DERIVATIVES

Acid	*$R_F \times 100$ solvent D*
Carolic	49
Carlosic	8
Terrestric	49
Viridicatic	8
Unknown ($C_9H_{10}O_5$)	49

SUMMARY

1. A sensitive spray reagent is described for the detection of some naturally occurring tetronic acids on paper chromatograms and electrophoretic strips.

2. New chromatography solvents are described and R_F values are reported.

3. The high voltage electrophoresis of tetronic acids is reported and their electrophoretic mobilities are recorded.

4. The identification of tetronic acids by means of their U.V. spectra is discussed; some relevant new data are recorded.

5. A paper chromatographic procedure is described for the partial characterisation of tetronic acids.

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CONTRIBUTION À L'ÉTUDE DES ACIDES ORGANIQUES DES MILIEUX BIOLOGIQUES

I. QUELQUES COLORATIONS DIFFÉRENTIELLES EN FONCTION DE LEURS CONCENTRATIONS

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(Reçu le 30 janvier 1961)

INTRODUCTION

Les acides du cycle de Krebs et tous ceux s'y rapportant, jouent un rôle très important dans le métabolisme général.

Par suite, on a cherché à obtenir une technique de chromatographie sur papier permettant de les séparer. Si ce problème est plus au moins résolu[1–5], leur identification et leur dosage ne l'est pas d'une manière satisfaisante.

Ces difficultés sont accrues par le fait que les indications des différents auteurs ne sont pas concordantes ni en ce qui concerne les couleurs obtenues, ni en ce qui concerne les seuils de détection.

Ces considérations nous ont amenés à tenter de trouver une méthode de détection et de dosage semi-quantitatif qui apparaisse plus simple que les méthodes ordinaires appliquées à la chromatographie sur papier telles que la titration potentiométrique, les dosages colorimétriques[6] et la polarographie[4].

En conséquence, il nous a paru intéressant de montrer les possibilités fournies par l'examen attentif des couleurs variées obtenues par les différentes concentrations des divers acides.

TECHNIQUE

Nous avons utilisé les solvants préconisés par NORDMANN et coll.[3,7]. Nous avons également obtenu de bons résultats avec ceux de OSTEUX ET LATURAZE[2].

Nous avons employé la technique chromatographique ascendante à deux dimensions (papier Whatman No. 3, 20 × 20 cm, enroulé en cylindre).

Les solutions étalons des acides ont été faites à 1 % dans l'alcool éthylique à 95 %, sauf pour les acides nicotinique (dans l'eau) et hippurique (dans l'alcool isoamylique).

L'étude a porté sur les acides organiques suivants (utilisés parce que habituels dans les milieux biologiques): acides aconitique, *cis*-aconitique, *m*-hydroxy-benzoïque, β-hydroxybutyrique, α-cétoglutarique, citrique, fumarique, glutarique, glycérique, glycolique, hippurique, lactique, maléique, malonique, nicotinique, oxalique, pyrrolidone-carboxylique, quinique[*], shikimique[*], succinique, tartrique. Nous avons ajouté les acides minéraux suivants: chlorhydrique, phosphorique, nitrique, et sulfurique car ils sont parfois incomplètement retenus par les résines.

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Les concentrations utilisées sont de 5 à 1500 γ. Les dépôts sont faits au moyen d'une séringue micrométrique "Agla".

Les différentes colorations employées ont été les suivantes:

1. Amine aromatique–sucre réducteur.
2. Diméthylaminobenzaldéhyde en milieu anhydride acétique.
3. Pyridine–anhydride acétique.
4. Nitrate d'argent.
5. Ferrocyanure de potassium.
6. Ferricyanure de potassium.
7. Réactif de BERG ET UFFELMANN.
8. Isatine.

Voici les techniques de colorations que nous avons employées:

1. *Amine aromatique–sucre réducteur*[8] *avec la modification de* HEITEFUSS[6]

Glucose	2 g
Aniline	2 ml

Dissoudre le glucose et l'aniline dans 20 ml d'eau, ajouter 20 ml d'alcool éthylique à 96° et 60 ml de *n*-butanol. Chauffer à 115° pendant 5 min dans une étuve bien ventilée.

Ce révélateur se conserve bien à l'abri de la lumière.

2. *p-Diméthylaminobenzaldéhyde en milieu anhydride acétique*[3,4,9]

p-Diméthylaminobenzaldéhyde	4 g
Anhydride acétique	100 ml

Ajouter à la solution quelques cristaux d'acétate de sodium. Après pulvérisation des chromatogrammes, chauffer pendant 3 min à l'étuve (130°).

3. *Pyridine–anhydride acétique*[10]

Pyridine	7 vol.
Anhydride acétique	7 vol.

Après pulvérisation des chromatogrammes, chauffer pendant 3 min à l'étuve (80–90°). Observer à la lumière du jour et à la lumière de Wood.

4. *Coloration du nitrate d'argent*[11], *modifié par* CARLES[12]

Solution d'ammoniaque	0.1 *N*
Solution de nitrate d'argent	0.1 *N*

Mélanger en parties égales. On peut ajouter au réactif une goutte d'acide acétique *N* pour chaque 5 ml de la solution de nitrate d'argent, pour éviter une précipitation très rapide.

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[*] Nous remercions très vivement Dr. JULES CARLES, du Laboratoire de Physiologie Végétale de l'Institut Catholique de Toulouse, qui nous a fourni ces deux acides.

Opérer à froid et laisser les chromatogrammes sécher à la température du laboratoire, à une lumière diffuse. Ensuite placer les papiers pendant quelques minutes à l'étuve aux environs de 40 à 50°. Observer les colorations à la lumière du jour et à la lumière de Wood.

5. *Coloration par le ferrocyanure de potassium*[12–14]

(a) Solution aqueuse de ferrocyanure de potassium à 10%
(b) Solution de sulfate ferrique d'ammoniaque à 0.5% dans l'alcool à 70°

Pulvériser les chromatogrammes avec la solution (a); sécher. Pulvériser avec la solution (b); laisser sécher. Noter les taches et leur différentes couleurs, en fond bleu clair (1 du Tableau I).

Placer alors les chromatogrammes dans l'étuve à 100°; le fond devient vert-bleu sombre. Décolorer avec de l'ammoniaque N.

Sécher et observer de nouveau les colorations (2 du Tableau I).

6. *Coloration par le ferricyanure de potassium*[12,15]

(a) Solution aqueuse à 0.3% de chlorure ferrique
(b) Solution aqueuse à 0.3% de ferricyanure de potassium

Mélanger extemporanément en volumes égales. Pulvériser les chromatogrammes.

Observer pendant la première heure; le fond est jaune et certaines taches apparaissent alors; laisser les chromatogrammes à la température du laboratoire et les observer 24 h après.

7. *Réactif de* BERG ET UFFELMANN[4,16,17]

Chlorure ferrique à 1% 100 ml
Acide phénique à 1%, q.s. jusqu'au violet persistant

Plonger les chromatogrammes dans le réactif et observer les couleurs au fur et à mesure qu'elles apparaissent avant de sécher, car il s'agit de couleurs fugaces.

8. *Réaction de l'isatine*[18,19]

Isatine à 0.2% dans l'acétone additionné de 4% d'acide acétique

Plonger les chromatogrammes dans la solution et les sécher à la température du laboratoire et ensuite, en étuve à 105° pendant 2 min. Observer les colorations.

RÉSULTATS

L'analyse du tableau et de la figure présentés nous permet de constater que tous les acides que nous avons utilisés se colorent avec l'aniline (sauf l'acide glycérique), quoique pas tous à la même concentration (10 γ selon LATURAZE). Ainsi, la plupart des acides se colorent avec des concentrations entre 10 et 50 γ, à l'exclusion des acides minéraux que se colorent à 5 γ (Fig. 1).

On peut aussi observer que tous les acides en question sont fluorescents dans l'U.V. avant que les chromatogrammes soient colorés, mais, d'une manière générale, seulement pour des concentrations plus fortes que celles qui s'avèrent nécessaires pour qu'ils soient colorés par l'aniline (Fig. 1).

TABLEAU

(Concentrations

Acides		*p-Diméthylaminobenzaldéhyde* (*Gaffney*)		*Pyridine–anhydride acétique* (*Furth*)		$FeCl_3$ + *acide phénique* (*Uffelmann*)	
		I	*F*	*I*	*F*	*I*	*F*
Adipique	1	350 J. fb.	—			150 Bi. fb.	350 Bi.
	2	700 Li. fb.	—	200 J. fb.	—		
Oxalique	1	700 J.	—			1000 J. cl.	—
	2	700 J. fo.	—				
Shikimique	1	200 Ro. cl.	700 Ro.			200 Bi. fb.	450 Bi. cl.
	2	350 Li. fb.	1000 Li. cl.	200 J. fb.	400 J.		
Quinique	1					50 J. Cl.	300 J. br.
	2	350 Fl. Bl.	—				
Malique	1	500 J. fb.	1500 J. B.			25 J. fb.	400 J. br.
	2	250 G. Li. fb.	700 J. B.	700 J. Bl.	—		
Tartrique	1	10 O. fb.	350 Ro. Vo. fo.	100 Bi. fb.	250 Bi.	10 J. fb.	200 J. br.
	2	50 Ro.	300 R. S.	50 Bl. J. fb.	200 J.		
Citrique	1	50 R. Vo. cl.	400 R. Vo. fo.	10 B. fb.	300 B.	50 J. fb.	350 J.
	2	50 Fl. V.	300 J. V.	5 Fl. V. cl.	200 J.		
Malonique	1	150 J. fb.	450 J.			200 Bl. fb.	350 Bl.
	2	300 J. cl.	450 J.	700 Fl. Bu.	—		
Nicotinique	1	250 J. O. fb.	350 J. O. cl.			150 Bi. fb.	450 Bi.
	2	350 J. O. fb.	—	150 J. Bl. fb.	1000 J. S.		
Glycerique	1						
	2						
Hippurique	1	10 O. cl.	400 O. fo.	5 J. cl.	500 R./J. +	250 J. Bi. cl.	400 Bi. cl
	2	10 J. cl.	50 J.	5 Fl. V. cl.	350 V. B.		
Maléique	1	350 J. fb.	—	300 B. fb.	—	350 Bi. cl.	450 Bi. O.
	2	500 J. fb.	—	50 J. fb.	300 J.		
Glycolique	1	700 J. B. cl.	—			25 J. fb.	200 J.
	2	700 J. fb.	1500 J. cl.	700 Fl. Bl. fb.	—		
Fumarique	1	50 Bi. fb.	250 Bi. cl.	10 J. B. cl.	500 Bi. fo.	50 Bi. fb.	400 Bi.
	2	50 B. cl.	400 B. Vo. fo.	5 Fl. J. S. cl.	300 B./J. +		

II

7)

$K_3Fe(CN)_6$		$K_4Fe(CN)_6$		$AgNO_3$		*Isatine*	
I	*F*	*I*	*F*	*I*	*F*	*I*	*F*
50 Bl. J. cl.	—	50 Li. fb.	—	25 B. Bl.	300 Bl./B. cl.	200 Li.	—
50		350 W. fb.	—	25 Fl. fb.	300 Fl. Vo. br.		
50 Bl.	150 Bl. br.	100 Bu. cl.	250 Bu. br.	150 B.	450 B. Bl.	150 Vo. Bu.	—
50 Bl. Bu.	—	100 Bu. W. fb.	300	400 Fl. cl.	—		
250 Bu. G.	—			50 Bi. cl.	350 B. Bi. fo.	100 Li.	—
300 Bu.	—	100 W. fb.	50 Bu. W.	50 Fl. fb.	300 Fl. cl.		
50 J.	—	25 Bu. fb.	300 Li.	150 B. cl.	350 B. R.	50 Li. fb.	400 Li. G. cl.
350 Li. V.	—	50 Bu. cl.	300 Bu. Vo.	500 Fl. fb.	—		
25 Bl.	—	5 Li.	200 Vo. fo.	10 B. fb.	300 Bl./B. G.	100 Li.	250 Vo.
25 Bl. J.	450 J.	50 Bu. cl.	—	25 Fl. fb.	300 Fl. Vo. cl.		
10 Bu.	250 J. cl.	5 Li. cl.	50 Li.	5 G. B.	350 Bl./B. fo.	150 Li.	—
400 Vo.	—	10 Bu.	300 Vo./Bu.	5 B. fo.	350 Fl. Vo. br.		
50 Bl. J. Bu.	—	50 Vo. fo.	—	10 Vo. cl.	250 B. fo./B.	150 Li.	—
400	—	50 W. Bu. fo.	—	50 Fl. fb.	150 Fl. cl.		
50 Bl.	—	25 Bu.	—	10 B. fb.	350 Bl./B. G.		
250 Bl. Bu.	—	25 W. cl.	300 W. Bu. fo.	25 Fl. fb.	350 Fl. Vo. br.		
100 Bl. J.	—			150 B. cl.	350 Ro./B. V.	250 Li.	—
250 V. Bu.	—	250 Bu. W. fb.	—	200 Fl. fb.	300 Fl. cl.		
100 J. cl.	1500 Bu. Bu. fo.						
50 Li. Vo.	1500 Li. Vo.						
50 J. fb.	—	500 W. Bu. fb.	—	150 Bl. B. cl.	250 B. Bl./B. cl.	400 Li. fb.	—
400	—	500	—	150 Fl. fb.	—		
100 J. cl.	—	50 Li.	350 Vo. Bu.	150 B./G. B.	300 B. + G. B.	250 Li. fb.	—
350 V.	—	50 W. J. cl.	—	150 Fl. fb.	—		
50 J. cl.	150 J. B. cl.	50 Li.	250 Li. Bu.	10 Ro.	300 Vo. fo.	50 Li. fb.	400 Li.
5 Vo.	200 Vo. W.	50 Bu. cl.	—	50 Fl. fb.	250 Fl. cl.		
25 J. cl.	200 J.	500 Li. fb.	1000 Li. Bu.	10 B. fb.	250 Bl./B. cl.	150 Ro. cl.	250 Vo.
25 Bu. cl.	300 Bu. J.	350 W.	—	25 Fl. fb.	250 Fl. Vo. br.		

(*Continué à p. 510*)

TABLEAU

Acides		*p-Diméthylaminobenzaldéhyde (Gaffney)*		*Pyridine-anhydride acétique (Furth)*		*FeCl$_3$ + acide phénique (Uffelmann)*	
		I	*F*	*I*	*F*	*I*	*F*
α-Céto-glutarique	1	50 Ro.	350 Ro. Vo. fo.	50 B. fb.	200 B. cl.	100 J. fb.	400 J. fo.
	2	50	350 R. S. fo.	50 Fl. Bl.	250 Fl. Bl. br.		
Aconitique	1	50 R. Vo.	350 Vo.	5 G. B. cl.	1000 G. N.	50 Bi. fb.	700 Bi.
	2	50 B. R. cl.	300 B. R.	5 Fl. V.	450 B.N. — V.		
cis-Aconitique	1	50 R. Vo.	350 Vo.	5 Vo. G.	350 Vo. G. fo.	200 Bi. fb.	200 Bi. cl.
	2	50 B. R. cl.	300 B. R.	5 Fl. V.	450 B.N. — V.		
Lactique	1	100 O. fb.	350 O. cl.	50 J. fb.	450 Ro. Li.	25 J. cl.	300 J. br.
	2	200 S. fb.	350 S. cl.	50 Ro.	400 R.		
Glutarique	1	250 J. fb.	—	350 B. fb.	—	200 Bi. fb.	350 Bi. O.
	2	350 J. cl.	—	150 J. fb.	300 J. S. cl.		
Pyrrolidone-carboxylique	1	450 Bi. fb.	1000 Bi. cl.	700 J. B. fb.	—	150 J. fb.	250 Bi. cl.
	2	700 J.	—	700 Fl. Bl.	—		
Nitrique	1	50 J.	700 J. B. fo.			1500 J.	—
	2	50 J. B.	850 B. Bi.	200 S. fb.	850 S.		
Succinique	1	250 Ro. fb.	700 Ro. cl.			150 Bi. fb.	700 Bi. Ro.
	2			150 Fl. fb.	1500 Fl. J. cl.		
β-Hydroxy-butyrique	1					350 Bi. fb.	—
	2			700 Fl. Bl.	—		
m-Hydroxy-benzoique	1	300 J. B. fb.	1500 Bi. cl.	700 J. B. fb.	—	150 Bi. fb.	350 Bi. J.
	2	300 J. fb.	1500 J. cl.	700	—		
Phosphorique	1	50 V. J.	—			50 Bl. fb.	350 Bl.
	2	50 J. fo.	—	350 J. fb.	—		
Chlorhydrique	1	50 J. br.	350 J. V. fo.				
	2						
Sulfurique	1	50 V. J.	250 V. J. fo.			150 Bl. fb.	—
	2	50 B. V. J.	250 B. V. J. fo.	100 Fl. Vo.	300 Vo.		

I = coloration initiale
F = coloration finale
Bi. = brique
J. = jaune
V. = vert
Ro. = rose
O. = orange

Bl. = blanc
B. = brun
G. = gris
Bu. = bleu
N. = noir
Vo. = violet
Li. = lilas

S. = saumon
R. = rouge
cl. = clair
br. = brillant
fb. = faible
fo. = foncé
Fl. = fluorescent

I *(continué)*

$K_3Fe(CN)_6$		$K_4Fe(CN)_6$		$AgNO_3$		Isatine	
I	*F*	*I*	*F*	*I*	*F*	*I*	*F*
50 Bl. Bu./Vo.	—	50 Li. fb.	350 Li. Bu.	150 B./V. fo.	400 B. fo./V. fo.	50 V. O. S.	350 V. O.
50 Bu. fo.	—	350 Bu. fb.	—	150 Fl. fb.	—		
10 J. B.	50 J.	50 Li. fb.	—	10 Ro. Bl.	400 Bl./B. G.	100 Ro. Li.	—
10 J. V.	—	250 V. fb.	—	10 Fl. Vo. fb.	350 Fl. Vo. R.		
50 J. B.	—	150 Li. fb.	—	10 Ro. Bl.	—	150 Li.	450 Vo.
200 V. cl.	—	350 V. fb.	—	10 Fl. Vo. fb.	—		
25 Bl. J.	50 J. cl.	50 Li. fb.	—	150 B. Bl.	—	250 Li.	—
25 Li./Bu.	450 Bu.	50 Bu. fb.	200 Bu. cl.	150 Fl. fb.	—		
50 J. fb.	—	50 Li. fb.	—	50 B. fb.	250 Bl./B. cl.	100 Vo. R.	400 Vo. R. fo.
350 V. fb.	—			50 Fl. fb.	250 Fl. Vo. br.	—	
50 Bl. J.	—	500 B. fb.	700 B. V.	150 Vo. Ro.	300 Ro. Vo.	100 Ro. cl.	350 Vo.
300 V./Bl. J.	—	400 V. fb.	—	150 Fl. fb.	—	—	
50 V.	—			250 B. J. fb.	350 B. J.		
25 Bu.	50 Bu. V.	50 Bu. V. fb.	—	250 Fl. fb.	—		
50 J.	—	50 J.	—	10 B. fb.	300 Bl./B. cl.	50 Ro.	350 Vo.
50 J. V.	400 J./Ro. fo.	200 V. cl.	—	10 Fl. Vo.	150 Fl. Vo. br.		
100 J. fb.	—			300 B.	350 B. fo.	250 Li. fb.	—
300	—						
150 J./Bu. fo.	—	250 Li.	—	150 B. cl.	350 Ro. Vo.	200 Li. fb.	—
500 V./Bu.	—	150 Bu. N. fb.	—	150 R. cl.	250 R.		
25 Bl.	—	50 Li./V. cl.	—	5 B. G.	300 Bl. J. V.	200 Li. fb.	—
25	500 Bl. J.	50 Bl.	150 Bl./V. cl.	5 N.	300 Fl. Vo. br.		
50 Bu. cl.	250 Bu. V. cl.	700 V. Bu.	—	25 B. Bl.	150 B. cl./G. V. fo.	250 Ro. fb.	400 Li.
300 Bu. V.	—			25 Fl. fb.	150 Fl. cl.		
150 Bl. J. fb.	—			10 Bl. J. fb.	300 Bl. Ro.	50 Li.	450 Vo. R.
200 V. fb.	—	200 V.	—	10 Fl. fb.	300 Fl. Vo.		

1 = lumière du jour } Gaffney, Furth
2 = lumière de Wood } et $AgNO_3$

1 = immédiatement } $K_3Fe(CN)_6$
2 = 24 h après }

1 = immédiatement } $K_4Fe(CN)_6$
2 = après l'étuve }

/ indique la présence d'une auréole autour de la tache
+ indique la zone plus marqué

En ce qui concerne les colorants appréciés dans leur ensemble (Tableau I), nous pouvons observer des différences de coloration très remarquables d'acide à acide, et même, pour chacun d'eux, des variations très grandes selon que l'on emploie l'un ou l'autre des colorants.

De plus, nous tenons à souligner quelques faits qui nous semblent dignes d'être observés:

Selon BUCH[20], l'acide fumarique ne provoque pas de coloration avec le colorant de FURTH alors que GODIN[21], LAMBOU[22] et LATURAZE[4], prétendent le contraire. Ce dernier indique même une concentration minimum (50 γ).

Nos résultats sont en accord avec ceux de ces trois derniers auteurs, mais nous trouvons un seuil plus bas que LATURAZE: 5 γ pour l'observation en U.V. et 10 γ pour l'observation à la lumière du jour.

Selon HALPERN[23], le colorant de BERG ET UFFELMANN colore l'acide glycolique mais non l'acide pyrrolidone-carboxylique. Or, nous avons obtenu des colorations avec les deux acides avec des seuils de 150 γ pour l'acide pyrrolidone-carboxylique et de 25 γ pour l'acide glycolique. L'affirmation du dit auteur pourrait être due au fait qu'il n'ait pas essayé diverses concentrations.

Les couleurs que nous avons obtenues sont en général en accord avec celles des autres auteurs. Nous avons cependant observé pour l'acide aconitique avec le colorant de FURTH une couleur gris cendré alors que LATURAZE obtient le jaune, et pour l'acide malique nous obtenons avec le nitrate d'argent une couleur châtaine alors que LATURAZE observe une couleur mauve.

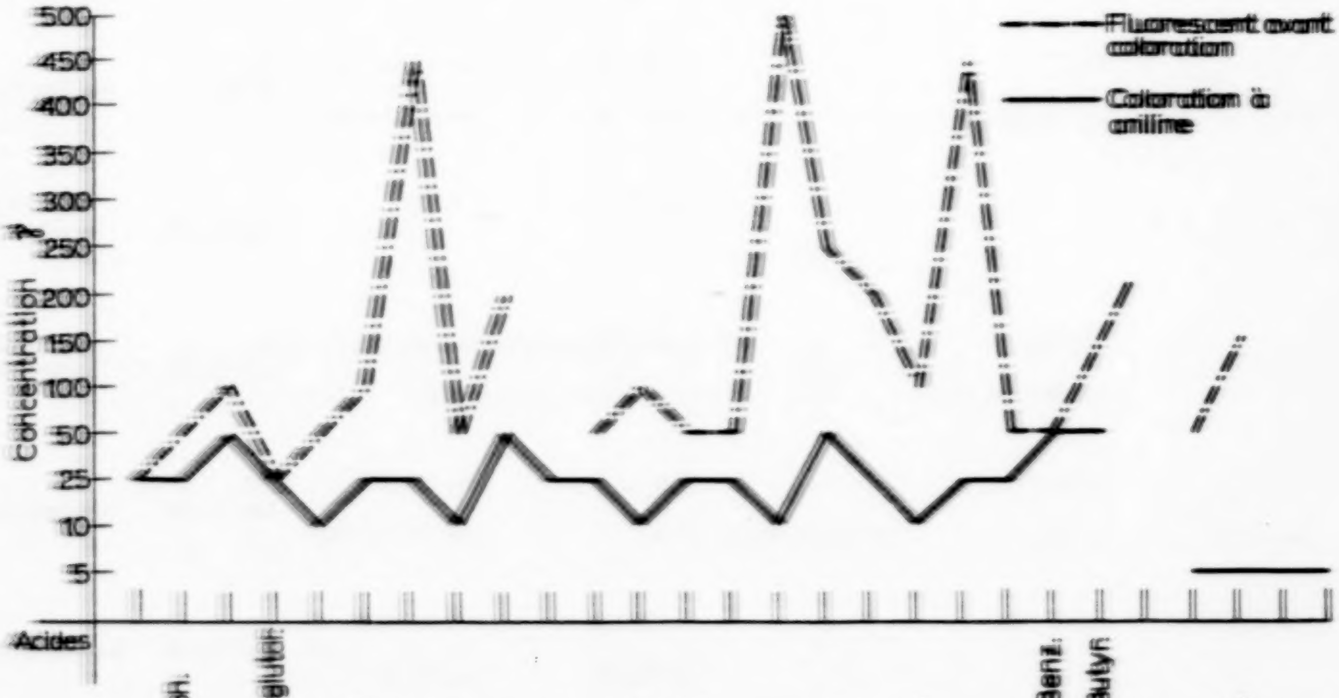

Fig. 1.

En résumé, nous ne prétendons pas avoir mis au point une technique permettant de caractériser dans tous les cas les acides organiques considérés, ni d'en indiquer les concentrations approximatives. Cependant en utilisant quelques colorants particuliers, l'étude du tableau et de la figure présentés facilitera certainement l'interprétation non seulement qualitative mais aussi, dans une certaine mesure, semi-quantitative de certains chromatogrammes.

REMERCIEMENTS

Nous remercions M. Osvaldo Antunes de sa collaboration technique.

RÉSUMÉ

Les auteurs ont mis au point une technique chromatographique pour la séparation et l'identification des principaux acides organiques des milieux biologiques.

La technique employée est: chromatographie bidimensionnelle ascendante, au papier Whatman No. 3 enroulé en cylindre, avec les solvants de Nordmann et coll., et en utilisant sept colorations différentes. La méthode permet la détection des acides organiques en question et leur appréciation semi-quantitative avec quelques colorants particuliers.

SUMMARY

The authors describe a chromatographic method for the separation and identification of the most important organic acids found in biological fluids.

The technique used involves two-dimensional ascending chromatography on Whatman No. 3 paper rolled into a cylinder, using the two solvents of Nordmann, followed by detection by means of seven different colour reactions.

With this method it is possible to detect the above-mentioned acids and, by using certain specific colour reactions, to carry out a rough semi-quantitative analysis.

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A NEW PAPER CHROMATOGRAPHIC SYSTEM FOR THE RESOLUTION OF 17-KETOSTEROIDS*

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(Received February 24th, 1961)

INTRODUCTION

A new Zaffaroni type of paper partition chromatography was developed for the resolution of the 17-ketosteroids (17-KS) found in urinary extracts[1,2]. Two disadvantages frequently associated with the Zaffaroni systems are that development times of several days may be required to insure adequate resolution of isomeric steroids and then a prolonged drying period is necessary to remove the stationary phase in order to satisfactorily perform the identification tests. A system which employs dimethyl sulfoxide (DMS) and *n*-heptane avoids these difficulties and represents a considerable improvement in several other respects.

EXPERIMENTAL

The solvents and reagents used were of the highest purity available. The steroids were purchased from Mann Research Laboratories and U.S. Pharmacopeia Reference Standards Collection. Stock solutions of steroids 1 mg/ml were prepared in 90% aqueous ethanol and stored in the cold. Dimethyl sulfoxide, b.p. 189–191° and *n*-heptane, b.p. 98–99°, were obtained from Matheson, Coleman and Bell. These solvents were equilibrated against each other prior to use. *n*-Heptane was employed as the mobile phase and 50 w/w% DMS (1 vol. DMS plus 1 vol. methanol) as the stationary phase. The ambient temperature was maintained at 24 ± 1.5°.

Whatman paper No. 1 which had been maintained at a relative humidity of 56% at 25° was used. These strips were dipped into 50 v/v% DMS and then blotted with paper towels. They were then dried between fresh paper towels for 2 h prior to sample application. From 10 to 200 µg of steroids were applied with micropipets on a 10 × 5 mm zone, 11 cm from the end of a sheet. The drying time and zone size were controlled by directing a stream of nitrogen under the point of application. The strips were placed in glass chromatographic tanks 12 × 12 × 24 in. which had been

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* This research was supported in part from grants made by the U.S. Public Health Service CY 3500 and the G. D. Searle and Co.

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pre-equilibrated with the solvents. Since the solvent-troughs contained 600 ml of mobile phase, a constant hydrostatic head could be maintained for extended periods.

Upon completion of the chromatographic run, the papers were inspected, and hung in a darkened fume-hood for an hour in order to remove most of the solvent. The sheets were then dried at 50° at 28 in. Hg overnight before being examined with ultraviolet light of 254 and 360 mμ wavelength, the Zimmerman test[2], the blue-tetrazolium test[3] and anisaldehyde–sulfuric acid–antimony trichloride reagent[4].

RESULTS AND DISCUSSION

The physical properties of dimethyl sulfoxide are consistent with the criteria established for a suitable stationary medium. It is a colorless neutral liquid which is transparent to U.V. light of 254 mμ wavelength. One can locate steroids containing a Δ^4-3-keto group by scanning the sheet immediately upon removal from the tank. The paper can be freed from the solvents by heating at 50° at 28 in. Hg for 12 h as compared to 18 h required for propylene glycol and several days for dimethyl formamide.

A system employing 50 v/v% DMS was found to be optimum as regards development time and ability to contain an adequate amount of sample. The use of higher concentrations increased the development time with no increase in loading capacity and resulted in a somewhat inferior resolution ability as compared to the standard system. The use of concentrations of DMS lower than 50% possessed no particular advantage while their capacity for steroids was inferior to the standard technique.

The rate of migration of the mobile phase is a function of the type of paper, hydrostatic head, ambient temperature, the concentration of the stationary phase and the drying time for the chromatographic strip after impregnation. A detailed study of the last factor indicated that the most reproducible results were obtained when the sheets were dried for 2.0 ± 0.5 h.

A 5° increase in the ambient temperature caused a 10–30% increase in the steroid mobility probably due to changes in the steroid partition coefficient. The relative mobilities for some typical 17-KS for both the *n*-heptane–DMS system and the ligroin–propylene glycol system (PML[5]) are assembled in Table I. The data, obtained at 24 ± 1.5°, are listed as relative mobilities with the reference steroids being deoxycorticosterone (DOC) and cortisone (CORT). Relative mobility is defined as the ratio of the distance travelled, per unit of time, by a given steroid to that of a reference steroid spotted on the same sheet.

The following program was used to resolve the following classes of 17-KS: compounds with an R_{DOC} of 1.0 or higher were developed for 2.5 h, while those with an R_{DOC} between 0.25 and 1.00 required a development time of 6–8 h, and steroids with an R_{DOC} of 0.20 or less 18–30 h, depending on the steroid*. Since DOC would have travelled off the sheet if used as reference for the last group CORT was used instead. The practice of employing a reference steroid was adopted not only to calculate relative

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* These data were obtained when the conditions were so adjusted that *n*-heptane required 2.5 h to travel 45 cm and the migration rate for DOC was 5.0 ± 0.2 cm/h.

TABLE I

A COMPARISON OF THE RELATIVE MOBILITIES OF 17-KETOSTEROIDS OBTAINED BY TWO PAPER CHROMATOGRAPHIC SYSTEMS

Ambient temperature was 24° ± 1.5° for the DMS system.

	Compounds	*Ligroin–propylene glycol*[5]	*Heptane–dimethyl sulfoxide*	
		R_{AND}*	R_{DOC}**	R_{CORT}***
1.	3α-Hydroxy-5α-androstan-17-one (androsterone)	1.00	2.28	
2.	3β-Hydroxy-5β-androstan-17-one	1.00	2.25	
3.	4-Androstene-3,17-dione	1.70	2.05	
4.	3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	0.70	1.85	
5.	3α-Hydroxy-5β-androst-9-en-17-one	0.70	1.80	
6.	3α-Hydroxy-5β-androstan-17-one	0.70	1.75	
7.	3β-Hydroxy-5-androsten-17-one (dehydroepiandrosterone)	0.70	1.35	
8.	5β-Androstane-3,11,17-trione	0.75	0.75	
9.	11β-Hydroxy-5α-androstane-3,17-dione	0.17	0.27	10.3
10.	3α-Hydroxy-5β-androstane-11,17-dione	0.26	0.20	6.7
11.	3α,11β-Dihydroxy-5α-androstan-17-one	0.10	0.12	3.5
12.	3β,11β-Dihydroxy-5β-androstan-17-one	0.048	0.09	2.25
13.	3β,11β-Dihydroxy-5α-androstan-17-one	—	0.075	2.15

* Mobility relative to androsterone as reference steroid.

** Mobility relative to deoxycorticosterone as reference steroid.

*** Mobility relative to cortisone as reference steroid.

mobilities, but also to establish whether the correct development time was used. Upon removal of a sheet from the chromatographic jar, it was inspected with a 254 mμ light source. If the reference steroid had travelled the correct distance, it would be removed for processing; however, if a longer development time was indicated, the run could be continued with no adverse effects.

The reproducibility of these data was established by a statistical analysis of 15 experiments in which the mobility of androsterone relative to DOC was compared. The mean R_{DOC} for androsterone was found to be 2.28 with a standard deviation of 0.15 or 7% of the mean. This is indicative of the order of reliability of the data. The major source of error is attributable to temperature fluctuations with its subsequent effect on the mobilities.

The ability of this system to separate steroids with almost identical mobilities cannot be explained solely in terms of small differences in distribution coefficients but rather as a manifestation of a mutual displacement effect[5]. This phenomenon occurs when two compounds with comparable mobilities migrate on the same sheet and one steroid will preferentially occupy all the sites thereby displacing the other and thus effect a physical separation. Illustrative of this is the experiment where a sample which contained 40 μg of 3α-hydroxy-5β-androst-9-ene, $R_{DOC} = 1.80$, 120 μg of 3α-hydroxy-5β-androstan-17-one, $R_{DOC} = 1.75$ and 40 μg of dehydroepiandrosterone, $R_{DOC} = 1.35$, were found to be distinctly separated from each other providing that the marker steroid, DOC, migrated 20 cm.

A comparison of the DMS system with the familiar ligroin–propylene glycol system (PML) is presented in Table I. The DMS system contained sets of 2 and 3 steroids with the same range of mobilities while the PML system contained 2 and 5 steroids, respectively*. With the DMS system the maximum development time was 30 h, while at least 7 days was required to achieve the same degree of resolution with the PML system. The latter system could handle a larger amount of steroid than the initial one. Up to 500 μg of crude urinary 17-KS have been processed successfully with the PML system; however, this amount of sample would overload the DMS system and cause considerable streaking.

These data supplemented with other unpublished results indicate that the DMS system is best suited for the resolution of steroids containing 1-3 oxygen substituents, present as hydroxyls or keto groups. The steroid esters, testosterone benzoate and androsterone benzoate, travelled with the solvent front and could not be resolved. Polar steroids such as corticosterone, cortisone, etc. were successfully resolved, but several days were required for development**.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Dr. William Metcalf for his encouragement and constructive criticism. Some preliminary data relevant to solubility of heptane in dimethyl sulfoxide were obtained by Mr. William Sharp, a summer student assistant. We are indebted to Dr. I. Goldring who generously lent us the apparatus necessary to construct a constant-temperature room during the course of this work.

SUMMARY

A paper partition chromatographic system for the resolution of 17-ketosteroids is described, which employs *n*-heptane as the mobile phase and dimethyl sulfoxide as the stationary phase. This system has several advantages over the Zaffaroni-types in current use, namely a development time of 2.5–24 hours for 17-ketosteroids, it affords excellent resolution, and the solvents used do not interfere with the characterization tests.

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Editor's Note: According to the referees the *R* values of compound 3 in Table I are doubtful.

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* It should be noted that all the steroids in both of these sets when present in mixtures, in reasonable concentrations, could be separated from each other by the DMS system apparently through the agency of the mutual displacement effect.

** At the time that this manuscript was being prepared for publication a report appeared indicating that organic acids could be resolved by a system similar to the one described here[6].

ADSORPTION CHROMATOGRAPHY ON CELLULOSE

VIII. THE BEHAVIOUR OF $SbCl_5$ IN VARIOUS CHROMATOGRAPHIC SYSTEMS

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(Received February 23rd, 1961)

In a previous paper[1], the remarkable increase in the adsorption of gold(III) halides on cellulose when chromatographed with mineral acids such as H_2SO_4 or H_3PO_4 was described. The phenomenon could not be elucidated with the results obtained with Au(III) halides and it was hoped that work with other halides which also adsorb strongly on cellulose would provide further evidence.

Unfortunately few halogeno-complexes are suitable for such studies[2]. Ga(III) and Po(IV) adsorb only from solutions containing high concentrations of HCl. There remained Sb(V) which in our studies gave consistently two spots due to the presence of hydrolysed species as well as $HSbCl_6$.

Thus, in order to study the adsorption of $HSbCl_6$ from, for example, H_2SO_4 solutions, a knowledge of the general solution chemistry and chromatographic behaviour of $HSbCl_6$ had to be obtained. The literature on the solution chemistry of $HSbCl_6$ consists of a detailed spectrophotometric study of equilibrium conditions and kinetics of hydrolysis[3,4] and a series of contradictory statements concerning the chromatographic behaviour. NEUMANN[3] claims that Sb(V) in HCl is not adsorbed on cation exchangers, while KRAUS *et al.*[5] use this very adsorption on Dowex-50 to effect separations of Sb(V) from other elements. The work of NEUMANN[3] shows clearly that a series of chloro-hydroxy complexes exist in all but 12 *N* HCl solutions and these have even yielded multiple spots in paper chromatography (MATSUURA[6]), while KRAUS AND NELSON[7] note one single species of Sb(V) in all concentrations of HCl in their study of the adsorption of metal ions on Dowex-1.

In our previous work on the adsorption of Sb(V) on cellulose we had observed two spots when working with Sb(V) obtained by oxidising Sb(III) in HCl[2]. In the present work we improved on the experimental conditions by replacing Whatman No. 1 paper by the thicker Whatman No. 3MM which permits chromatography with conc. HCl and by using pure $SbCl_5$ as the starting product.

By carrying out "progress chromatograms" with solutions of $SbCl_5$ in various concentrations of HCl in water, confirmation (although qualitative) of the spectrophotometric work of NEUMANN[3] was obtained. In very dilute HCl (3 *N* and 1 *N*) the hydrolysis of $HSbCl_6$ is considerably slower than expected, compared to higher concentrations, but this is also in agreement with the kinetic studies of NEUMANN[4] on the effect of H^+ ions on the speed of hydrolysis.

Partition and ion exchange chromatograms carried out with solutions of varying degree of hydrolysis were then considered. Paper chromatography with butanol–6 *N* HCl or butanol–3 *N* HCl yields essentially the same chromatograms irrespective of the starting solution used, thus suggesting considerable alteration of the complexed state during development with organic solvents. The slow-moving spot described by MATSUURA[6] was absent in these chromatograms although we had observed it on previous occasions with solutions of Sb(V) which were obtained by oxidation of Sb(III). This would indicate that the slow-moving spot is possibly due to polymers formed when oxidation is carried out in dilute HCl, since the simple hydroxy-chloro complexes are present in our solutions.

Ion exchange studies with Whatman anion exchange resin paper yielded a good resolution of the various chloro-hydroxy Sb(V) ions, giving good correlation with the spectrophotometric results of NEUMANN[3].

From the above studies it was evident that during a chromatographic run of about 20 to 40 min a complete hydrolysis of $HSbCl_6$ does not occur even in very dilute HCl. Several days are necessary for the complete decomposition of $HSbCl_6$ in 1 *N* HCl. Thus unless an unlikely specific catalytic effect is exerted by aqueous H_2SO_4 or H_3PO_4 on $HSbCl_6$, any increase of adsorption due to these acids in the developing solvent must indicate the formation of compounds between these acids and $HSbCl_6$ in a way which does not involve the loss of a chloro-group from the coordination shell of the Sb(V). As is shown in the experimental part, there is a marked increase of adsorption from mixtures of HCl–H_2SO_4 as well as from either H_2SO_4 or H_3PO_4.

EXPERIMENTAL

(1) *Studies with solutions of $SbCl_5$ in HCl by adsorption chromatography on cellulose*

Solutions of $SbCl_5$ in HCl were prepared by adding 1 ml of pure $SbCl_5$ (Carlo Erba, Milano) to 100 ml of HCl of the required strength at room temperature (17 ± 1°). Solutions in 100%, 90%, 80%, 70%, 60%, 30% and 10% conc. HCl were prepared. The solution in 10% HCl yields a bulky white precipitate while the others form clear solutions, which have a straw-coloured tint in higher concentrations of HCl.

Fig. 1 shows a progress chromatogram of the solutions from 100% to 60% conc. HCl being chromatographed several minutes, 2 h, 17 h and 24 h after addition of the $SbCl_5$ to the HCl solutions. 50% aqueous HCl was used as solvent. The solution in conc. HCl yields a faint forward trail with this developing solvent; however, since the concentration of HCl is below that of all the solutions in which hydrolysis is being followed, the reconversion of hydrolysed species into $HSbCl_6$ cannot occur.

In Fig. 2 some results with solutions of $SbCl_5$ in 30% and 10% HCl are shown using 30% HCl as developing solvent. Even in 30% HCl, $HSbCl_6$ shows little tendency to hydrolysis during development. It is interesting to note that the $HSbCl_6$ spot will still be obtained from solutions in 10% HCl for several days.

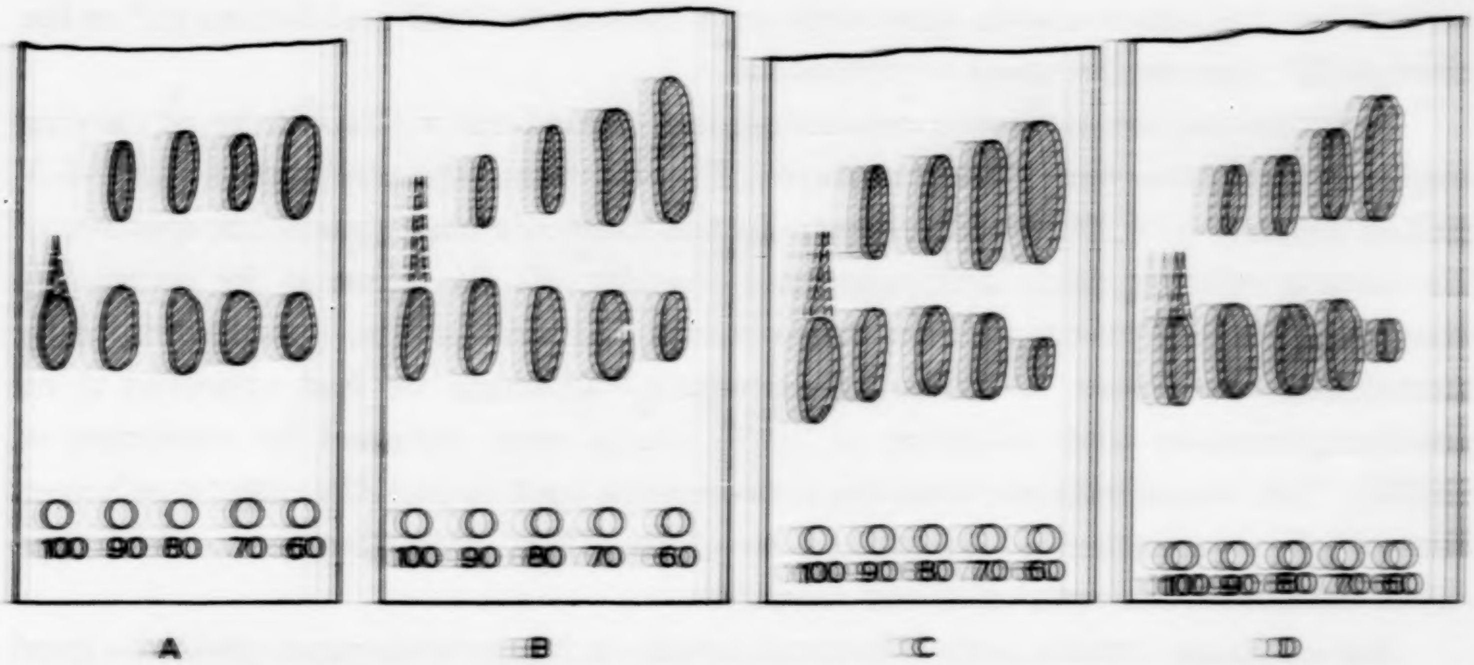

Fig. 1. A progress chromatogram of pure $SbCl_5$ dissolved in conc. HCl, 90%, 80%, 70% and 60% aqueous HCl. Paper: Whatman No. 3 MM; solvent 50% aqueous HCl. The spots are revealed by spraying with an aqueous KI solution. (A) Chromatographed a few minutes after adding $SbCl_5$ to the HCl solution; (B) the same solutions 2 h later; (C) the same solutions after standing 17 h; (D) after 24 h.

The R_F value of the hydrolysed species increases in the dilute HCl solutions but not enough to permit a separation from the slower hydrolysed species.

(2) *Partition chromatography*

The five solutions chromatographed in Fig. 1 were run on Whatman No. 3MM paper with butanol–3 *N* HCl and butanol–6 *N* HCl (equal volumes). In butanol–3 *N* HCl all solutions gave a spot at R_F 0.52 (with reference to the solvent front) another at 0.79 (on the acid front) and a third spot on the solvent front. The spot on the solvent front is strongest with $SbCl_5$ in conc. HCl. In butanol–6 *N* HCl all solutions gave a double

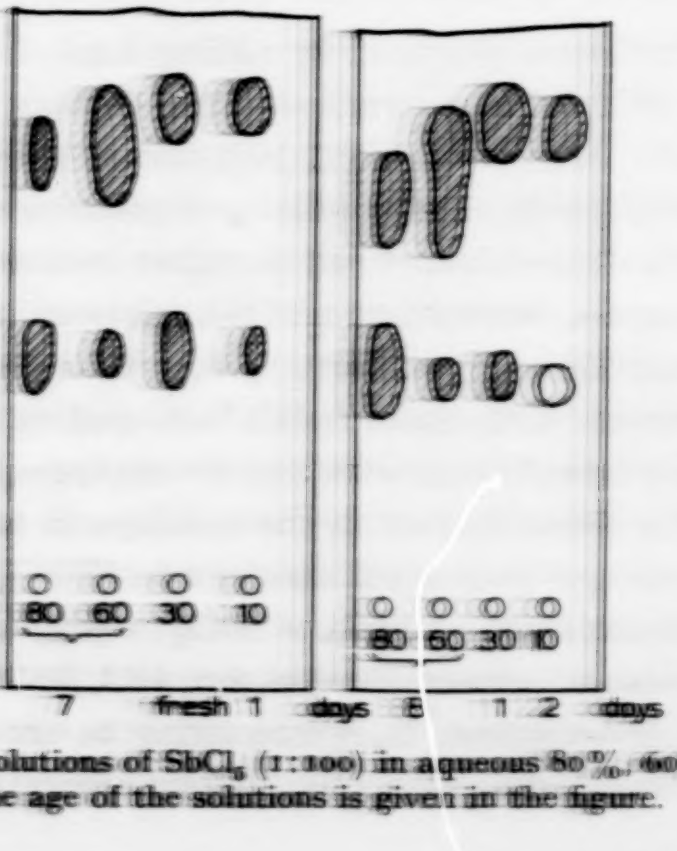

Fig. 2. Chromatograms of solutions of $SbCl_5$ (1:100) in aqueous 80%, 60%, 30% and 10% HCl. The age of the solutions is given in the figure.

spot on the liquid front and just below it. Owing to this establishment of equilibrium in the organic solvent, partition chromatography seems unsuitable for analysing solutions of $SbCl_5$ in HCl.

(3) *Ion exchange chromatography*

Whatman "Weak Anion Resin Loaded Paper" kindly supplied by its manufacturers was used, and development was carried out by the ascending method. Fig. 3 shows some of the results with 50% and 30% HCl as developer. If these are compared with the separations on cellulose paper and/or the results obtained by spectrophotometry[3] it is obvious that both $HSbCl_6$ and $HSb(OH)Cl_5$ are strongly retained at the origin. $HSb(OH)_2Cl_4$ moves with an R_F of 0.36 (approximately) in 30% HCl and only 0.04 in 50% HCl. Hence also for this species the adsorption is not one of ion exchange, otherwise adsorption would have to be stronger with a more dilute HCl solution. In aged solutions of $SbCl_5$ in 30% and 10% HCl, another spot at the liquid front can be detected which could evidently be due to $HSb(OH)_3Cl_3$ and more hydrolysed species. It is thus possible to detect $HSbCl_6$ on cellulose paper, and to separate mixtures of hydrolysed species into three groups on resin-loaded paper.

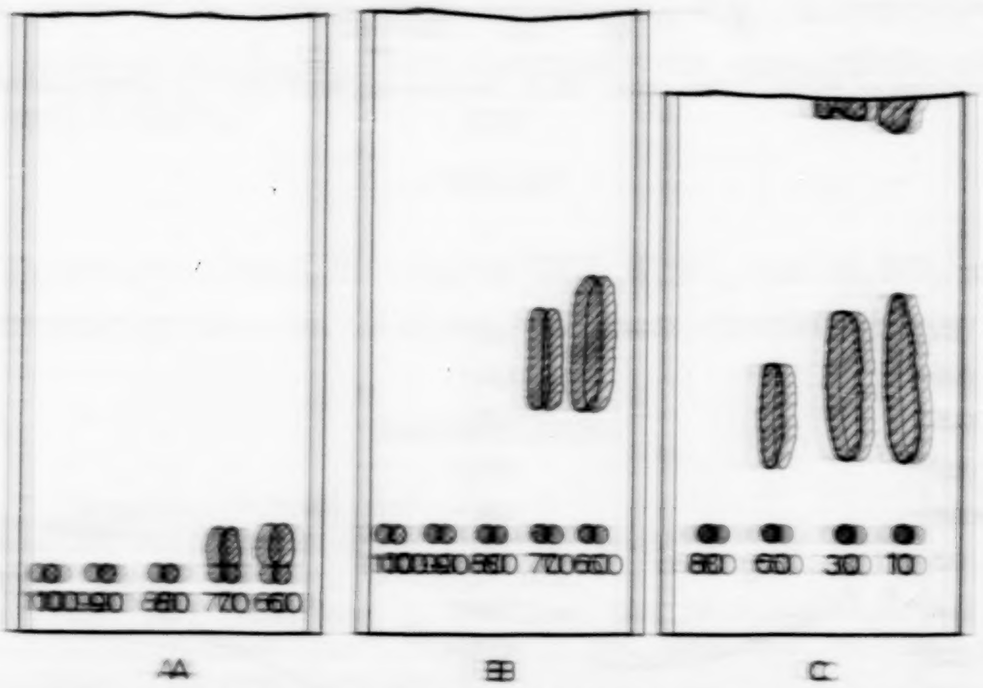

Fig. 3. Chromatograms of aged solutions of $SbCl_5$ in HCl (concentrations given under each chromatogram in the figure) on Whatman "Weak Anion Resin Loaded Paper". (A) With 50% HCl as solvent, (B) and (C) with 30% HCl as solvent.

(4) *The effect of H_2SO_4 and H_3PO_4 on the adsorption of $HSbCl_6$ on cellulose*

$HSbCl_6$ in conc. HCl was studied throughout, as this was shown to contain practically pure $HSbCl_6$ without hydrolysis products.

Fig. 4 shows the R_F values obtained when mixtures of HCl and H_2SO_4 are used as developing solvent, and these are compared with the R_F values in aqueous HCl. Even in water, N/10 and N/2 HCl no extensive decomposition of the $HSbCl_6$ spot could be observed in a 10 cm run which requires about 20 min, although a forward comet

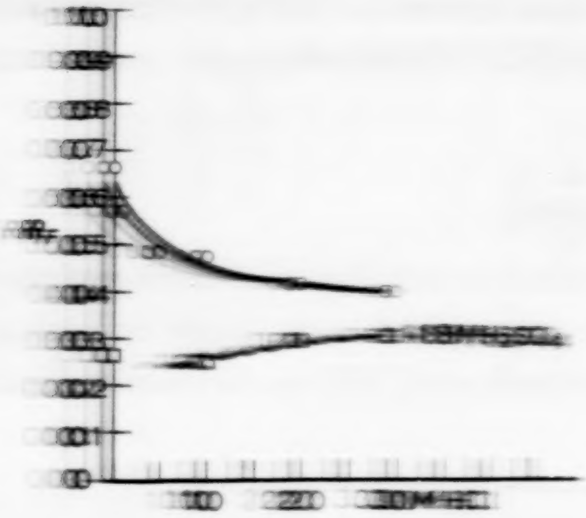

Fig. 4. The R_F values of $HSbCl_6$ (in conc. HCl) developed with HCl or mixtures of 18 M H_2SO_4–HCl.

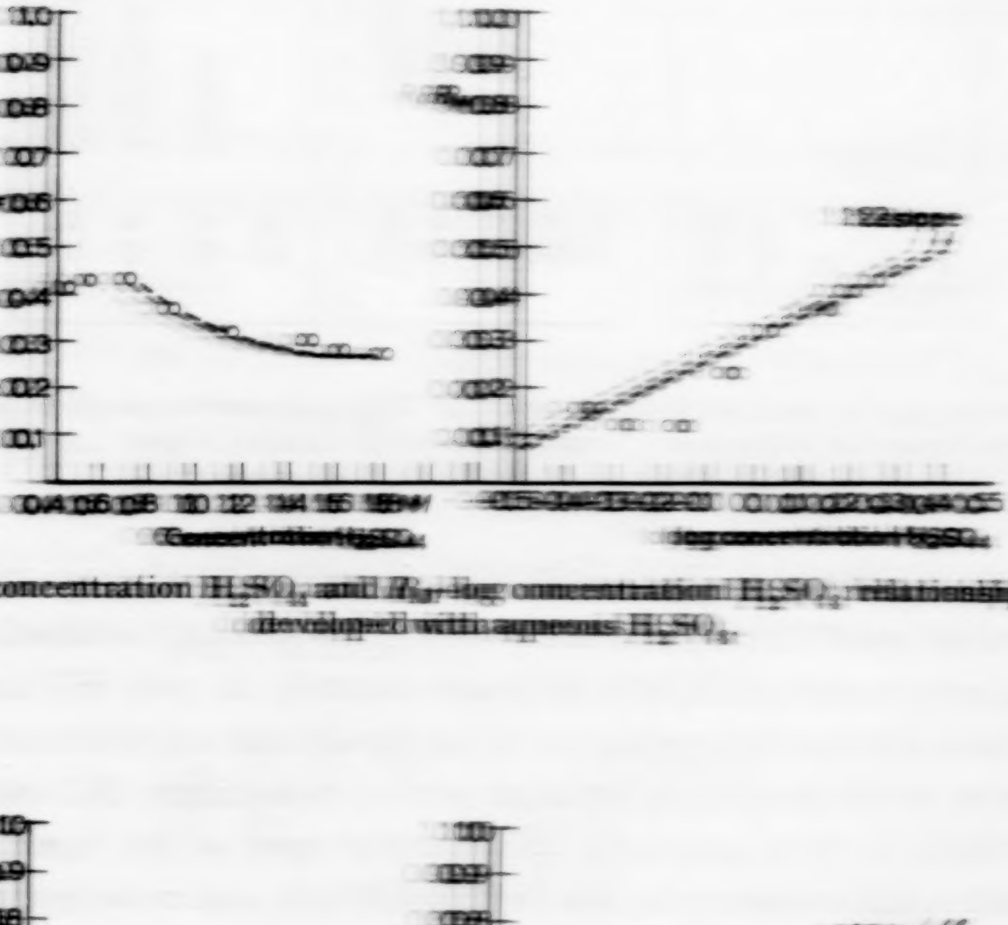

Fig. 5. R_F-concentration H_2SO_4 and R_M-log concentration H_2SO_4 relationships for $HSbCl_6$ developed with aqueous H_2SO_4.

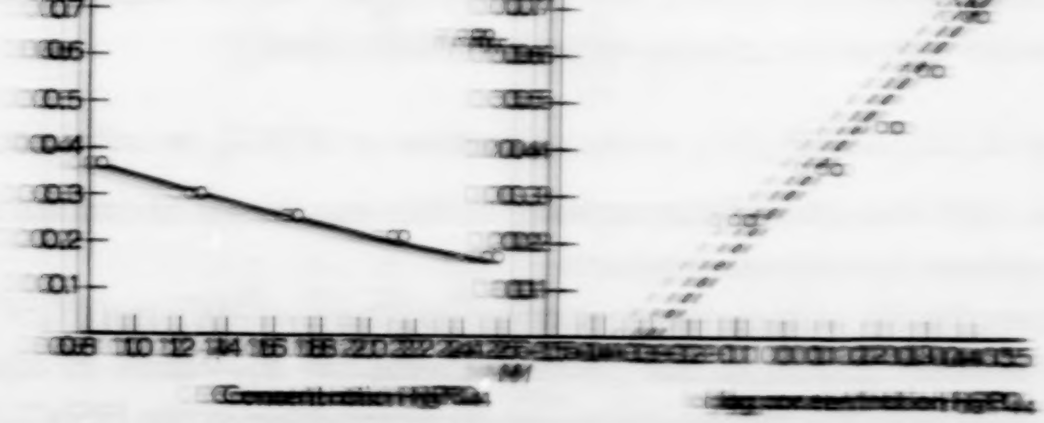

Fig. 6. R_F-concentration H_3PO_4 and R_M-log concentration H_3PO_4 relationships for $HSbCl_6$ developed with aqueous H_3PO_4.

and sometimes a faster spot clearly indicates that hydrolysis products are being slowly formed.

Figs. 5 and 6 show the R_F-concentration and R_M-log concentration relationships for $HSbCl_6$ developed with H_2SO_4 or H_3PO_4. In H_2SO_4 the hydrolysis is quite considerable, however, the $HSbCl_6$ spot is always clearly distinguishable. The change of the R_F values follows closely that observed with $HAuCl_4$. In dilute (less than 1 N) acids the results with $HSbCl_6$ in conc. HCl are not highly accurate as the conc. HCl influences the movement of the spot of the Sb(V) solution over quite a distance.

For the data with solutions more concentrated than 1 N (with respect to H_2SO_4 or H_3PO_4) there seems to be a 1:1 slope for the R_M-log concentration relation for H_3PO_4 and a 1:2 slope for H_2SO_4. Thus essentially the same effects are obtained as with $HAuCl_4$ and $HAuBr_4$.

SUMMARY

The solution chemistry of $SbCl_5$ in aqueous HCl, H_2SO_4 and H_3PO_4 was studied by adsorption chromatography on cellulose, partition chromatography and ion exchange.

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PAPER CHROMATOGRAPHY OF INORGANIC IONS

XIX. THE CHROMATOGRAPHY OF POLONIUM AND OTHER IONS ON ACETYLATED FILTER PAPER

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(Received March 12th, 1961)

INTRODUCTION

Work with milligram amounts of polonium has shown that reduction of Po(IV) with SO_2 yields a pink solution of Po(II) (in dilute HCl) which is auto-oxidised to Po(IV) within a matter of minutes[1]. It thus seemed of interest to us to attempt paper chromatographic experiments with Po(II) since here only tracer amounts are required and hence the behaviour of Po(II) in the absence of the auto-oxidation effect of its α-rays may be observed.

In the usual partition chromatography with butanol-HCl mixtures no differences in R_F values could be observed between chromatograms run in air and in SO_2, although this problem was investigated carefully[2]. Furthermore no difference between Po(II) and Po(IV) was noted in adsorption paper chromatography with aqueous HCl solutions, where Po(IV) is strongly adsorbed[3]. It was, however, possible to distinguish between Po(IV) and lower valencies of Po by chromatography with organic solvents on acetylated cellulose paper, a technique which has not been used previously for inorganic ions.

This paper will describe the behaviour of a number of inorganic ions on acetylated paper and more specifically the differences obtained when polonium is chromatographed with air and with CO_2 and SO_2 as atmosphere and after mixing with reducing agents.

EXPERIMENTAL

(1) *Chromatography on acetylated paper*

Throughout this work either Binzer No. 202 acetylated paper with 45% acetylation or Schleicher and Schüll paper with 20–25% acetylation was employed. All the acetylated papers are hydrophobic so that development with aqueous solutions is impossible.

Solutions of ions to be chromatographed are best made in butanol–HCl mixtures since aqueous solutions do not wet the paper.

Table I shows the R_F values of several metal ions when developed with butanol–HCl mixtures on ordinary and acetylated papers.

(2) *The behaviour of Po(IV) on acetylated paper*

Solutions of Po(IV) in HCl were chromatographed in butanol–HCl mixtures on Schleicher and Schüll paper (20–25% acetylated). As shown in Fig. 1, Po(IV) is strongly retained on the acetylated paper, thus behaving like the chlorides of Au(III), Fe(III), Tl(III) and Sb(V) (which, incidentally, are also extracted into ether).

TABLE I

R_F VALUES OF SOME CATIONS ON ACETYLATED FILTER PAPER

Cations	R_F values on	
	Whatman No. 1	Binzer (25% acetylated) paper
Solvent I: butanol–conc. HCl (2:1)		
Au(III)	1.0	*0.20–0.28*[*]
Bi(III)	0.53	0.58
Cd(II)	0.89	0.65
Cu(II)	0.62–0.39	0.55
Fe(III)	1.0	*0.23*
Hg(II)	0.86	0.63
Tl(III)	1.0	*0.35*
Sb(III)	0.76	*0.41*
Se(IV)	0.68	0.60
Ni(II)	0.14	0.18
Ga(III)	1.0	*0.27*
Sb(V)	1.0 comet to 0	*0.15* forward comet
Al(III)	0.07	0.16
Co(II)	0.63	0.63
Mn(II)	0.15	0.28
Solvent II: butanol–8 N HCl (2:1)		
Au(III)	0.95	*0.15*
Cd(II)	0.89	0.83
Co(II)	0.24	0.38
Cu(II)	0.50	0.49
Fe(III)	1.0	*0.44* comet to 0.04
Hg(II)	0.87	0.73
Ni(II)	0.19	0.37
Solvent III: butanol–6 N HCl (2:1)		
Au(III)	0.95	*0.14*
Cd(II)	0.97	0.94
Co(II)	0.24	0.40
Cu(II)	0.40	0.46
Fe(III)	1.0	*0.38* comet to 0.08
Hg(II)	0.95	0.85
Ni(II)	0.21	0.34

[*] Figures in italics indicate a great difference between the two papers.

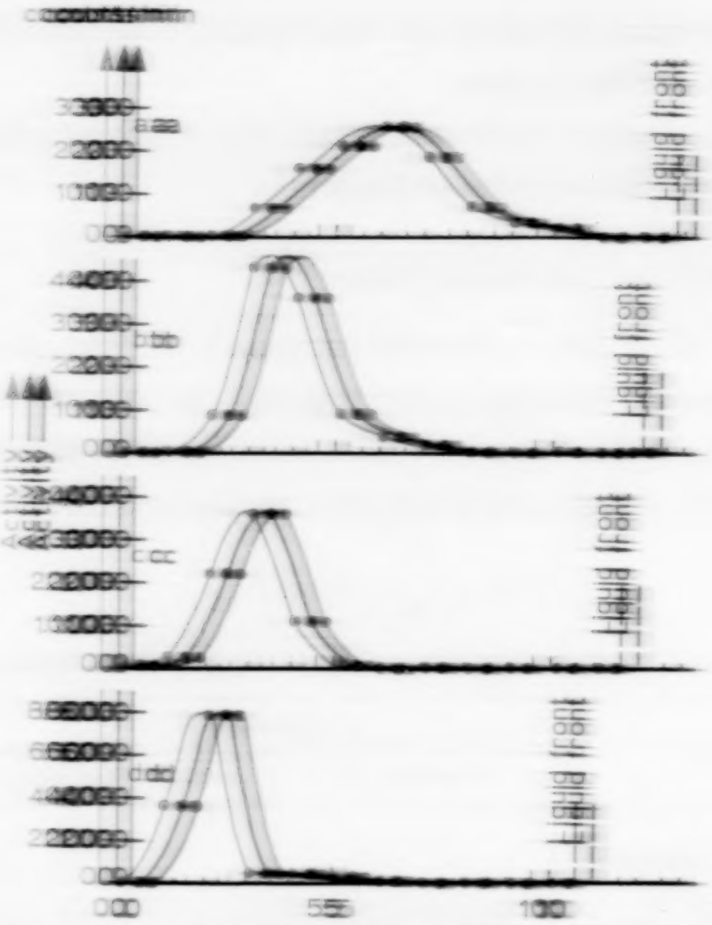

Fig. 1. Activity distribution on chromatograms of Po(IV) chromatographed in air (or CO$_2$) on Schleicher and Schüll 20–25% acetylated paper. Solvents and R_F values: (a) butanol–6 N HCl (2:1), R_F 0.47; (b) butanol–8 N HCl (2:1), R_F 0.31; (c) butanol–10 N HCl (2:1), R_F 0.28; (d) butanol–12 N HCl (2:1), R_F 0.21.

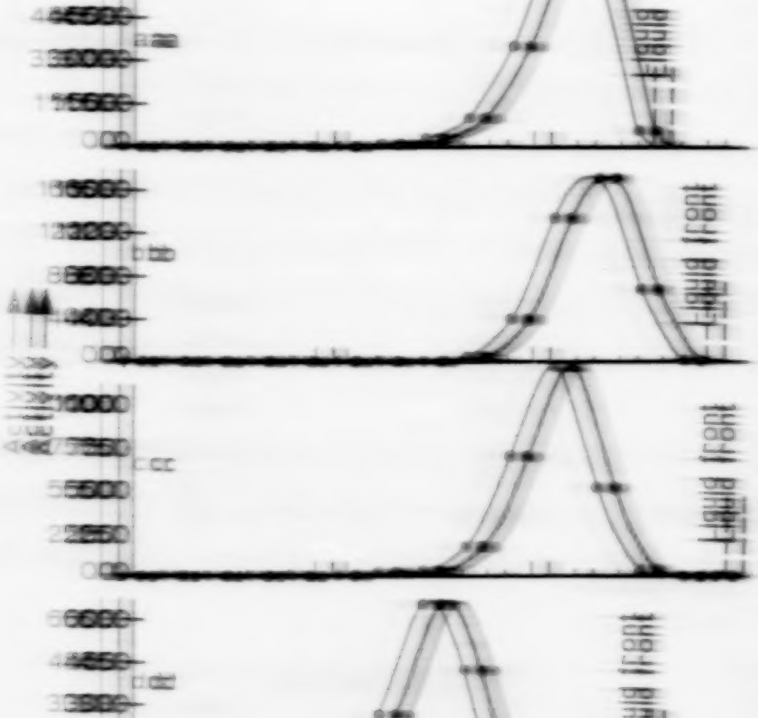

Fig. 2. Activity distribution on chromatograms of Po (originally as Po(IV)) chromatographed in an SO$_2$ atmosphere on Schleicher and Schüll 20–25% acetylated paper. Solvents and R_F values: (a) butanol–6 N HCl (2:1), R_F 0.84; (b) butanol–8 N HCl (2:1), R_F 0.80; (c) butanol–10 N HCl (2:1), R_F 0.72; (d) butanol–12 N HCl (2:1), R_F 0.61.

(3) *Experiments on the reduction of Po(IV) and chromatography in an inert or reducing atmosphere*

Po(IV) when chromatographed in an SO_2 atmosphere in the apparatus of [illegible]* moves close to the liquid front when developed with butanol–HCl mixtures as shown in Fig. 2. According to BAGNALL[5] the species chromatographed was Po(II) and we have thus found a chromatographic system in which Po(IV) and Po(II) can be readily distinguished. We then proceeded to examine the influence of various reducing agents and of the atmosphere on this chromatographic system. When Po(IV) was mixed with cuprous chloride in HCl and chromatographed in a CO_2 atmosphere, usually two spots were obtained of which one corresponds to Po(IV) and the other moves faster than Po(IV) but slower than Po(II).

Typical results are shown in Fig. 3. The presence of Po(IV) indicated that the

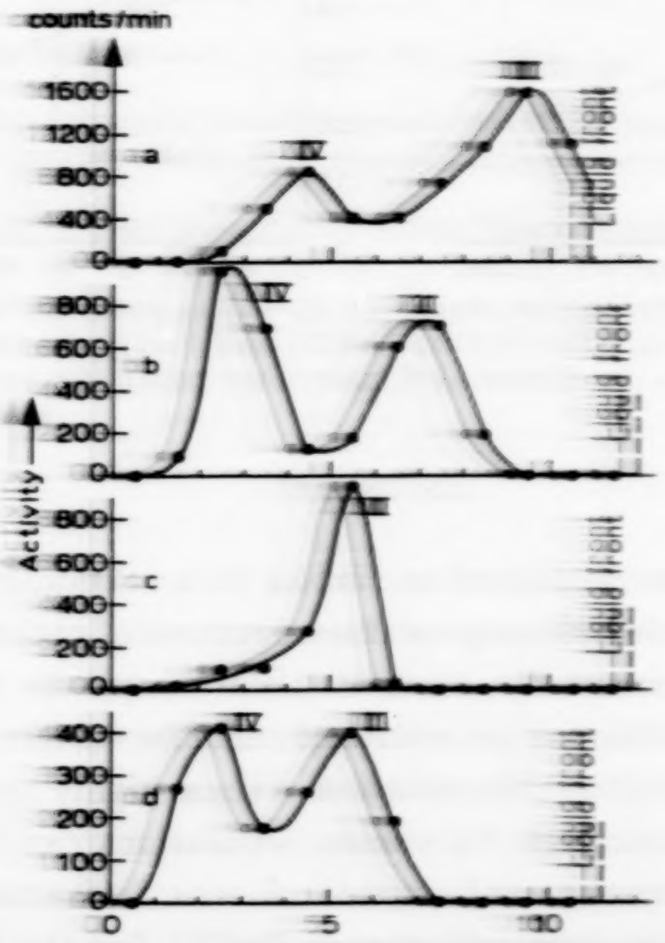

Fig. 3. Activity distribution on chromatograms of Po(IV) mixed with Cu(I) chromatographed in a CO_2 atmosphere on Schleicher and Schüll 20–25% acetylated paper. Solvents and R_F values: (a) butanol–6 N HCl (2:1), R_F 0.41 and 0.88; (b) butanol–8 N HCl (2:1), R_F 0.23 and 0.60; (c) butanol–10 N HCl (2:1), R_F 0.46; (d) butanol–12 N HCl (2:1), R_F 0.21 and 0.49.

reducing conditions led to a partial reduction and the intermediate spot should thus correspond to Po(III), a valency also observed in the auto-oxidation of macro-amounts of Po(II) by BAGNALL AND FREEMAN[1], but whose existence could not be confirmed by other methods.

The spot of intermediate R_F value was also obtained when Po(IV) was reduced with Fe(II), SO_2 or Ti(III) and chromatographed in an inert (CO_2) atmosphere. This behaviour would be in agreement with the oxidation potential of about 0.74 V, which can be deduced from the results of BAGNALL AND FREEMAN[1]. The behaviour of Po(II), Po(III) and Po(IV) is summarised in Fig. 4. On ordinary cellulose paper (*e.g.* Whatman No. 1) there is no difference between the movement of Po(III) and that of Po(IV) in butanol–HCl mixtures and, as has been observed previously[2], Po(II) also cannot be distinguished.

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* An apparatus described in the handbook of HAIS AND MACEK[4].

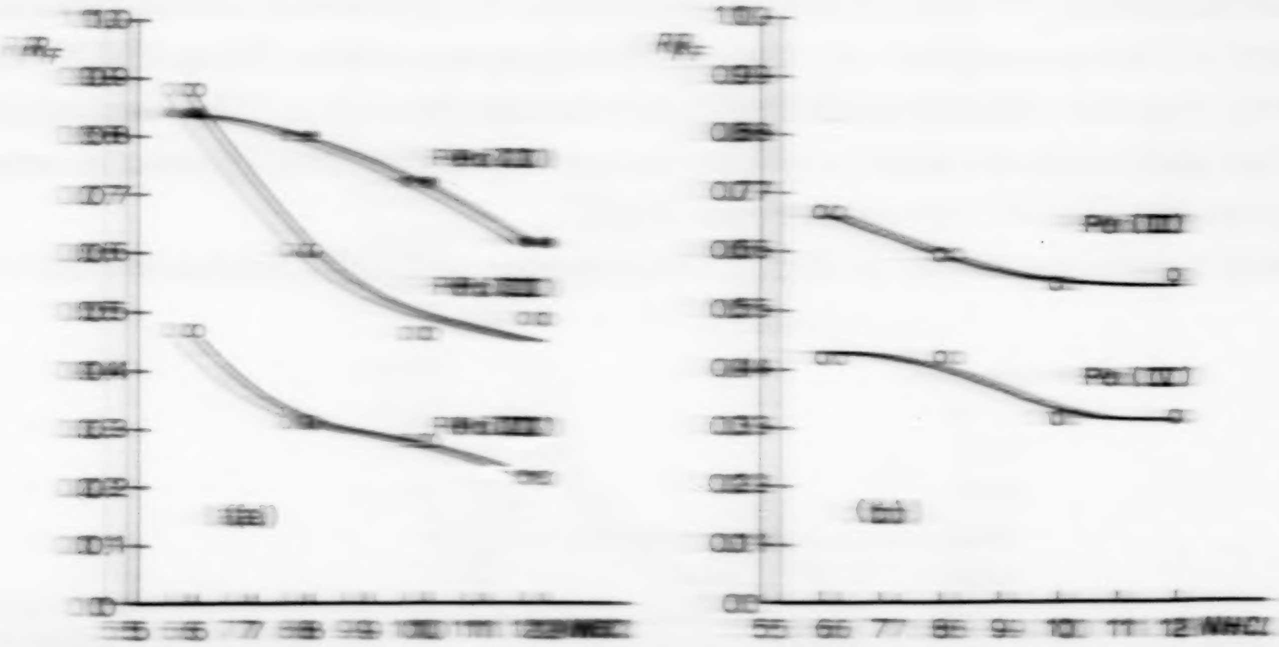

Fig. 4. Graphical representation of the change of R_F values with the concentration of HCl in butanol–HCl mixtures ratio [illegible] for Po(IV), Po(III) and Po(II). (a) On Schleicher and Schüll 20–25% acetylated paper. (b) On Binzer 45% acetylated paper (for Po(IV) and Po(III) only).

DISCUSSION

Acetylated paper must be considered as similar to a stationary highly oxygenated liquid phase, which has little influence on the movement of cations and some complex anions, but extracts preferentially those halogen complexes which are soluble in ethyl ether and are also adsorbed on resin and cellulose surfaces. While in the usual partition chromatography little differentiation of these highly hydrophobic complexes may be made owing to their high R_F values, separations can be achieved by chromatography on acetylated papers with organic solvents. In the usual paper chromatography no differences can be observed between Po(IV) and the lower valencies of Po which all travel with the liquid front. On acetylated cellulose, three differently moving species could be observed in butanol–HCl mixtures: rather strongly adsorbed Po(IV), practically unadsorbed Po(II) which exists only in an atmosphere of SO_2, and a species obtained by the addition of reducing agents to Po(IV) and chromatography in air or CO_2, which has R_F values intermediate between Po(IV) and Po(II).

It is unlikely that this latter species is an equilibrium mixture of Po(II) and Po(IV), because both Po(IV) and this species may be separated from each other. A gradual oxidation of Po(II) to Po(IV) during the development should result in a trail and not a well-defined zone as is actually the case. As this species is formed under milder reducing conditions than Po(II) and is still stable in air, we consider it likely that it may be Po(III) which, according to the work of BAGNALL AND FREEMAN[1] should be formed under these conditions. Our observations seem to constitute a confirmation of these results and the first chromatographic isolation of Po(III).

Previous evidence for the existence of Po(III) obtained by several workers by co-crystallisation experiments (for a summary see[5]) was considered doubtful by Bagnall[5] because the oxidation of Po(III) [illegible] as well as the ready reduction by Fe(II) [illegible] the presence of Po(III) in these experiments is not excluded.

SUMMARY

The chromatographic behaviour [illegible] on acetylated paper with butanol–HCl was investigated. The three valencies of polonium may be differentiated in this system.

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ANION EXCHANGE SEPARATION OF THORIUM FROM URANIUM

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(Received March 13th, 1961)

The first comprehensive study of the adsorption of the elements by strong base anion exchangers was reported for hydrochloric acid media by KRAUS AND NELSON[1] at "The First International Conference on the Peaceful Uses of Atomic Energy" at Geneva, Switzerland, in 1955. These authors as well as HUYS[2] showed that thorium cannot be adsorbed on such resins even at the highest possible hydrochloric acid concentration, because of the low tendency of the thorium ions to form negatively charged chloride complexes. The adsorption of uranyl ions, on the other hand, shows a steady increase with increasing hydrochloric acid concentration of the solutions, so that separation of thorium from uranium could be achieved in such media, although a simultaneous adsorption of thorium and uranium was out of the question.

Previous research work by KORKISCH AND TERA[3] in the field of anion exchange in mixed solvents, has shown that thorium tetrachloride can be adsorbed on strongly basic resins from solutions containing aliphatic alcohols and hydrochloric acid. This adsorption, however, is only high if aliphatic alcohols of greater chain length than methanol are applied in high concentrations (85-95 %). The highest adsorption coefficients of thorium on Amberlite IRA-400 could be obtained in mixtures consisting of 90% propyl-, butyl- or amyl alcohol and 10% hydrochloric acid. Since, under the same conditions, uranyl ions are also very strongly held, a simultaneous adsorption of both these elements is possible.

In the present paper the distribution coefficients of thorium and uranium between butanol-, methanol-HCl mixtures and Dowex 1 were determined. The results showed that both these elements have very high distribution coefficients in butanol-HCl mixtures. In methanol-HCl mixtures, however, only uranium is held on the resin with sufficient strength thereby making possible a separation of these two radio elements from each other. By means of column operations thorium and uranium can thus be adsorbed simultaneously on the resin from a butanol-HCl mixture. By treating the exchanger with a methanol-HCl mixture thorium is eluted whereas uranium still remains adsorbed. Afterwards the elution of uranium can be achieved by means of 1 N hydrochloric acid.

EXPERIMENTAL PART

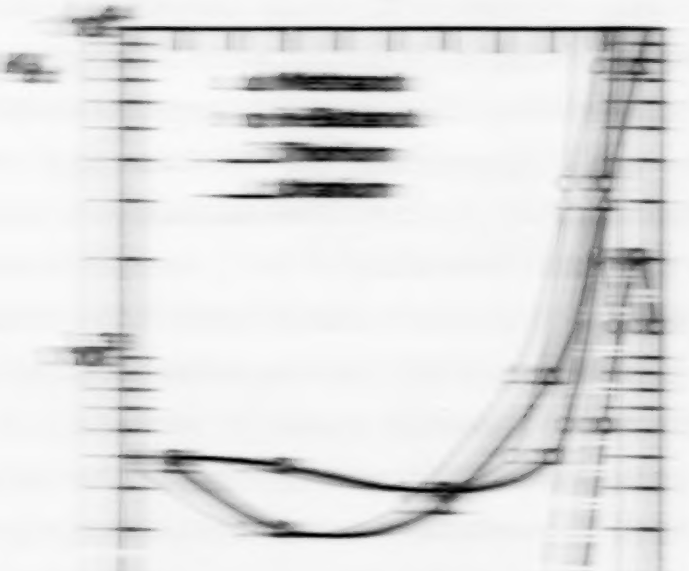
Solutions and reagents

Analytical Dowex 1, X8 (100-200 mesh, chloride form) was used. The aliphatic alcohols (methanol and n-butanol) were reagent grade solvents. The standard solutions of

[illegible]

[illegible]

[illegible]


0
10
20
30
40
50
60
70
80
90
100

[illegible]

Determination of distribution coefficients

The distribution coefficients of uranium and thorium were always determined in mixtures of 25 ml volume containing 5000 µg thorium or uranium.

Example: For mixtures containing 96% butanol or methanol, 1 ml of either the uranium or thorium standard solution (containing 5000 µg thorium or uranium per ml) was mixed with 24 ml of the corresponding alcohol, using a conical flask. After preparing the solution, 1 g of resin was added, and the stoppered flask was shaken thoroughly on a shaking machine for 24 h. The resin was then filtered off, and the thorium or uranium was determined in the filtrate as described above. The results shown in Fig. 1 were obtained using this procedure.

Column operations (separation of thorium from uranium)

After filling the ion exchange column with resin suspended in methanol the resin bed was pretreated portionwise with in total 50 ml of a mixture consisting of 45 ml *n*-butanol and 5 ml 6 *N* hydrochloric acid. Then the sorption solution (45 ml *n*-butanol + 5 ml 6 *N* hydrochloric acid) containing the thorium and uranium was passed through the column at a flow rate of 15 ml/h. The column was then washed (flow rate 30 ml/h) with 150 ml of a mixture consisting of 90% methanol and 10% 6 *N* hydrochloric acid. During this step the thorium is eluted and can be found up to 90% in the first 50 ml of the effluent. The other 50 ml contained the residual amount of thorium, while in the last 50 ml no trace of thorium could be detected. At the same time the uranium remains strongly adsorbed on the resin (5000 µg uranium occupy a resin zone of less than 1 cm height, which is practically not shifted during the elution of thorium). Afterwards the uranium was eluted with 100 ml 1 *N* hydrochloric acid.

All separations of thorium from uranium were completely quantitative (see Table I), owing to the fact that the distribution coefficient for thorium in the methanol–HCl medium employed for elution is 7, whereas that for uranium is 3000.

RESULTS

Fig. 1 shows the effect of the *n*-butanol and methanol concentration on the distribution coefficient K_d which is given by the following equation:

$$K_d = \frac{\mu\text{g thorium (uranium)/g resin}}{\mu\text{g thorium (uranium)/ml solution}}$$

From this figure it can be seen that the distribution coefficient of thorium and uranium increases with increasing alcohol concentration. From 90% alcohol (methanol or butanol) upwards very high values for uranium were obtained. In this figure the distribution coefficients for uranium in 99% and 96% butanol and 99% methanol are not shown, in order to avoid extending the scale of the figure further. The corresponding distribution coefficients are 833,300, 75,750 and 12,100 respectively.

The curves for thorium show that this element has the highest distribution coefficients in 80–99% butanol only, whereas at all methanol concentrations its distribution coefficient is rather low. These great differences in the distribution coefficients of thorium and uranium in methanol–hydrochloric acid medium are the basis for the separation method described.

In Table I the results of a series of separation experiments (see experimental part) are recorded. From these results it is seen that this method of separation proves to be quite suitable for the separation of micro- and semi-macro quantities of thorium and uranium.

TABLE I

SEPARATION OF THORIUM AND URANIUM

Amounts taken		*Amounts recovered*	
thorium μg	*uranium* μg	*thorium* μg	*uranium* μg
10	10	11	9.8
100	100	105	102
1000	1000	1010	999
5000	5000	5040	5012
50	5000	50	5020
5000	50	5040	49
10	5000	11	4980
5000	10	5060	10.5

This method could be applied preparatively for the continuous separation of the thorium isotopes that are formed by the natural decay of uranium. In this case the column loaded with uranium has to be washed every now and then with the methanol–hydrochloric acid mixture employed for the separation of thorium from uranium. The effluent will contain the thorium isotopes formed during the time that has passed between two successive washings.

ACKNOWLEDGEMENTS

The research reported above, was sponsored by the International Atomic Energy Agency and the United States Atomic Energy Commission under contract number 67/US. The generous support from these agencies is gratefully acknowledged.

SUMMARY

The present work was carried out in order to develop a method for separating thorium and uranium. Equilibrium studies have shown that the adsorption of both tetravalent thorium and hexavalent uranium as negatively charged chloride complexes on the strongly basic anion exchanger Dowex 1 from solutions containing butanol and hydrochloride acid is very high, so that these two radio elements can be adsorbed on the resin simultaneously. Separation is possible due to the fact that thorium can be readily eluted with a methanol–hydrochloric acid mixture, whereas uranium is still very strongly retained on the resin under the same conditions. After the removal of thorium the uranium is eluted with 1 *N* hydrochloric acid.

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Short Communications

Use of an asphalt fraction for the gas–liquid chromatography of steroids

The rather high temperatures which have to be employed for the gas–liquid chromatography (GLC) of steroids have until recently restricted the choice of stationary phases to a small number of thermostable substances. Non-polar phases resistant to high temperatures are not difficult to find, and successful applications of the method, using Apiezon and the silicones, have been made for the separation of sterols differing in the number of carbon atoms or in configuration.

For the separation of more closely related sterols, for example pairs differing only by one double bond, a polar stationary phase is necessary, and such phases are notoriously thermolabile. BEERTHUIS AND RECOURT[1] separated cholesterol and cholestanol by converting them into the acetates and chromatographing on a polyester. The disadvantages of this method for routine use are the necessity for a preliminary acetylation of the mixture and the fact that polyesters are markedly unstable at temperatures above 190°, so that one is forced to accept relatively long retention times.

Recently VANDENHEUVEL, HAAHTI AND HORNING have described[2] a new polar stationary phase, QF-1, which is a fluorinated alkyl silicone and as such is both polar and stable at high temperatures. They noted a separation of cholesterol and cholestanol using this material, and obtained a separation factor of 1.09. We had meanwhile discovered that the already known phases[3] Apiezon L and SE-30, which when used alone are incapable of separating these two sterols, may be successfully modified to do so by the addition of a fraction obtained from air-blown asphalt. This fraction has the necessary separating properties because of its content of high-molecular-weight condensed aromatic rings.

The fraction is obtained by repeatedly extracting the asphalt, first with *n*-hexane and subsequently with benzene–absolute ethanol (1:1, v/v). The residue is readily soluble in benzene and in chlorinated hydrocarbons, but virtually insoluble in petroleum ether, ether, and ethyl acetate. It is not suitable for use as such for GLC since its melting point is too high and the consequent high viscosity leads to "tailing". At the temperatures we employ (about 240°) the asphalt fraction is apparently held in solution by Apiezon or SE-30; the viscosity is reduced and the tailing avoided.

The column filling found thus far to be the most suitable for our purpose, and which is resistant to temperatures up to 260° for long periods, consists of Chromosorb P (Johns-Manville) which has first been made hydrophobic with dimethyl-dichlorosilane and subsequently impregnated with 1–4 % (by weight) Apiezon L or SE-30 and 0.5–1 % of the asphalt fraction described. Appropriate amounts of both substances are dissolved in benzene, and the mixed solution is applied to the support in the usual manner. Columns containing Apiezon and the asphalt fraction gave longer retention times for the steroids than those employing SE-30 as diluent. Some separations of steroid mixtures are shown in Figs. 1 and 2. The apparatus consisted of a "Pye" Argon Chromatograph. The samples (10–20 μg) were introduced as follows: a glass rod provided with a small metal spring was dipped into the steroid solution. After evaporation of the solvent this rod was brought into the column.

The use of Chromosorb P as supporting medium led to the remarkable observation that sterols have the same retention times as their acetates or benzoates,

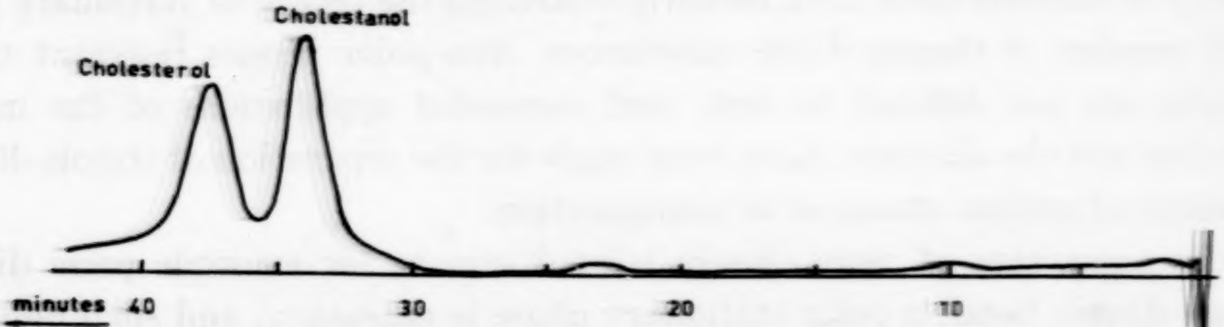

Fig. 1. Separation of cholesterol and cholestanol by gas–liquid chromatography, using Apiezon L and an asphalt fraction (see text) as stationary phase. Column 140 cm; Chromosorb P, 1.5% Apiezon L, 0.8% asphalt fraction; 250°; 2 atm.

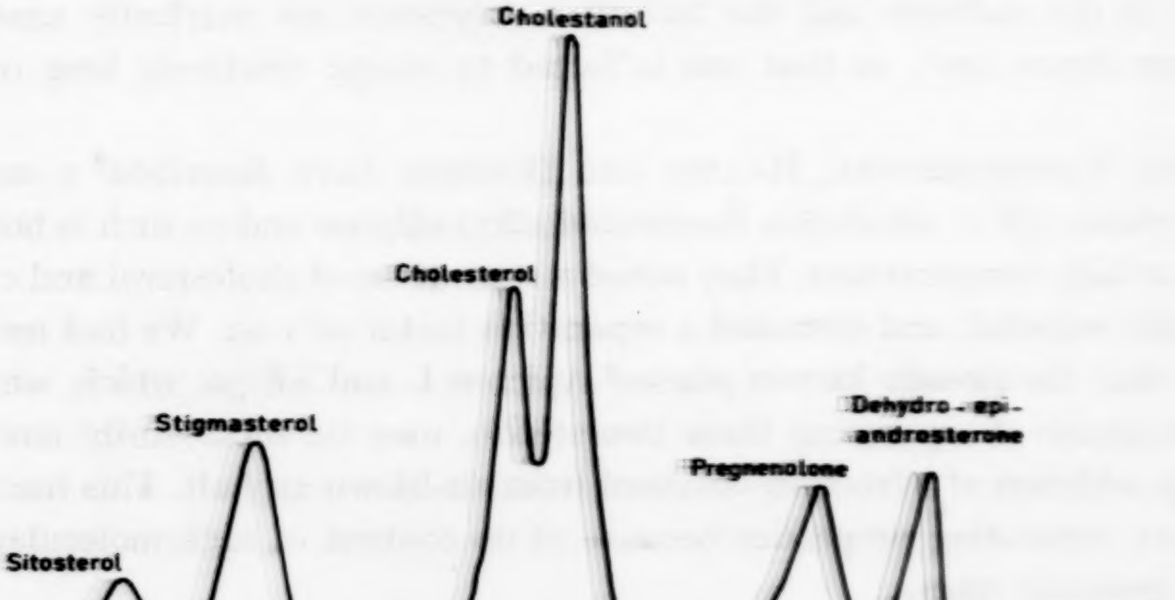

Fig. 2. Steroid GLC separations using SE-30 and the asphalt fraction as stationary phase. Note shorter retention times of cholestanol and cholesterol. Column 140 cm; Chromosorb P, 4% SE-30, 0.4% asphalt fraction; 240°; 2 atm.

irrespective of the stationary phase. By trapping the fractions leaving the column and subjecting them to "thin-layer" chromatography (on silica) and infra-red spectroscopy, it was possible to show that Chromosorb P strongly catalyses the removal of hydroxyl and acyl groups from sterols and sterol esters respectively to yield compounds with a new double bond, probably between C_2 and C_3. Thus cholestanol and cholestanyl acetate both yield a cholestene, while cholesterol and cholesteryl acetate (or benzoate) yield a cholestadiene. The fact that this transformation takes place both quantitatively and virtually instantaneously at the top of the column was revealed by "thin-layer" chromatography and by the symmetrical shape of the peaks obtained from a mixture of cholestanol and cholesterol. It appears that the cholestene and cholestadiene are not further decomposed during their passage through the column, and that they are quickly and efficiently separated in the presence of the asphalt fraction.

The same dehydration and de-acylation on this support has been observed with sterols with shortened side-chain, for example pregnenolone, *epi*-dehydroandrosterone, and their acetates. The catalytic effect was also shown, though to only a small extent, when the Chromosorb P was replaced by acid- and ammonia-washed Celite 545. In the presence of the polyfluorethylene support "Haloport F" (F. and M. Scientific Corp., New Castle, Del., U.S.A.) all trace of the transformation had disappeared, but it was not possible to achieve satisfactory plate numbers with this material. The most successful combination remained SE-30, the asphalt fraction and Chromosorb P; because of the quantitative nature of the dehydration or de-acylation such columns are perfectly suitable for quantitative analysis.

This investigation was made with the support of the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).

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Received August 15th, 1961

Paper chromatographic separation of sennoside A and sennoside B

Sennosides A and B, the active glucosides of senna, are known to differ in their biological activity and sennoside B is reported to be much more active than sennoside A[1]. The separation of the two stereoisomeric compounds is quite important for the evaluation of the activity of the crude drug and its extracts and the determination of the individual active glucosides.

By using a modified paper chromatographic technique of RUTTER[2,3], we have achieved the separation of the two sennosides. In the technique, a large filter (18 cm diameter) was employed, and as a developing "wick", a strip of paper just enough for the purpose (1.5 cm in length and 2 mm in width) was cut from the center and bent towards it so that it was kept perpendicular to the plane of the paper.

Separation of the two compounds was accomplished by using two solvent systems, *viz.* (I) upper layer of solvent mixture from *n*-butanol–glacial acetic acid–water (40:10:50) and (II) upper layer of solvent mixture from *n*-butanol–dilute acetic acid (1.93 *N*)–water (40:10:50) for developing. Revelation was achieved by spraying the dried paper with 2% alcoholic sodium hydroxide solution. The glucosides developed a yellow color with this solution and the free 1,8-dihydroxyanthraquinone derivatives a pink color. The yellow color of the sennosides changed gradually to brown after about a day. The average R_F values of sennosides A and B in solvent I are 0.79 and 0.68 respectively and in solvent II are 0.68 and 0.45 respectively.

By using the solvent system II which was found to be definitely better than solvent I for the separation of the glucosides, it was also possible to separate the free 1,8-dihydroxyanthraquinone derivatives, *viz.* chrysophanol, aloe-emodin, emodin and rhein from each of the two glucosides. It was observed that these compounds which also occur in senna, moved almost to the solvent front with R_F value of 0.93 when the solvent system II was employed.

The separation of the free anthraquinones from sennosides was confirmed by chromatography of 70% alcoholic extract of senna (*Cassia angustifolia* Vahl) pods. In connection with this procedure used for confirmation, it might be mentioned that chromatography of this crude extract of senna pods revealed the presence of six bands other than those due to the two glucosides and free anthraquinones when solvent system II was used for development and alcoholic alkali was used for spraying. Further work on this aspect is in progress.

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Received September 8th, 1961

Notes

A peak simulator for testing pen recorders

Gas chromatography columns and detectors commonly give peculiar chromatographs when operated under unsuitable conditions or when fed with unsuitable sample material. In these circumstances persons untrained in fault analysis require simple test apparatus to be available.

Since the pen recorder is sometimes suspect, the instrument shown in Fig. 1 has been designed to enable easy performance checks to be made on the recorder after it has been isolated from the remaining apparatus.

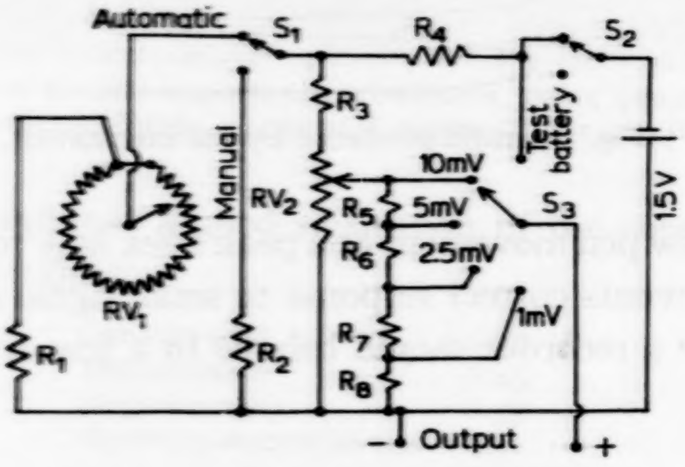

Fig. 1. Diagram of the instrument.

Fixed resistors, all 1/2 W ± 5%:	R_1	22 Ω	R_5	50 Ω
	R_2	1000 Ω	R_6	25 Ω
	R_3	470 Ω	R_7	15 Ω
	R_4	4700 Ω	R_8	10 Ω

Variable resistors: RV_1 5000 Ω; high resolution; linear.
RV_2 100 Ω; linear (sensitivity control).

Switches: S_1 Single pole 2-way rotary switch.
S_2 Single pole 2-way rotary switch.
S_3 Single pole 5-way rotary switch.

Electric motor: "Sectric" Light Duty Motor, type 200; 2 rev./min, (Smiths Clocks and Watches, Ltd., London).

Provision has been made for two methods of testing. With S_1 switched to "Manual", if RV_2 is rotated very quickly, servo-mechanism "overshoot", "undershoot", and response time may be observed. S_3 may also be used for this test.

RV_1 is employed when S_1 is switched to "Automatic". RV_1 is a high resolution potentiometer with a metal track connected between the ends of the resistance windings, so that the slider arm may continue to make electrical contact. The slider is continuously rotated by a small electric motor at some convenient and constant speed, the resistance values of the whole instrument being selected to load RW_{11} in such a way that the volts per degree of arc swept by the motor shaft vary according to the position of the arm. Since a linear track potentiometer is used, the resulting peak is symmetrical, with the metal track providing a base line which is maintained at a few percent of recorder full scale deflection by R_{11}.

Several pen recorder faults may be detected; the fast deflection produced at the beginning and end of a peak allows recorder "noise" and response to fast inputs to be observed, while the slow pen movement at a peak apex may reveal alternating current interference which prevents correct response to small signal changes.

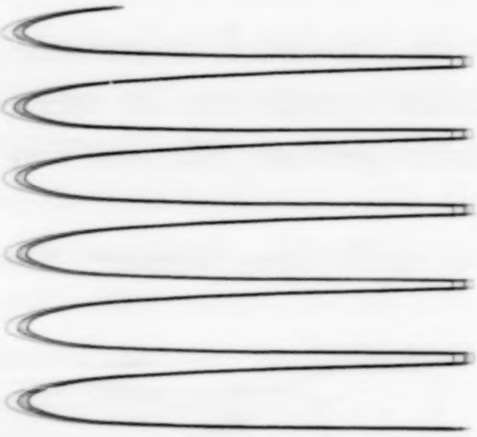

Fig. 2. Graph produced by the instrument.

Fig. 2 shows how a recorder should behave to a few cycles of operation of this instrument.

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Received June 21st, 1961

A new solvent system for the paper chromatographic separation of some pharmacologically active carbamates

We have developed a new solvent system for the paper chromatographic separation of the pharmacologically active carbamates meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate), carisoprodol (N-isopropyl-2-methyl-2-propyl-1,3-propanediol dicarbamate), mebutamate (2-methyl-2-*sec.*-butyl-1,3-propanediol dicarbamate)[1] and hydroxymeprobamate (2-methyl-2-β-hydroxypropyl-1,3-propanediol dicarbamate)[2]. Previous solvent systems employed for this purpose, such as butanol–acetic acid–water (4:1:5, v/v/v), give almost identical values for the first three compounds[3,4].

The solvent mixture employed consists of carbon tetrachloride–acetic acid–water

(1:2:1, v/v/v). The lower organic phase is utilized at 11° in a descending manner on Whatman No. 1 paper for 6 h. Color development is carried out using a modification of the method of RYDON AND SMITH[5] which consists essentially of exposure to chlorine fumes followed in ten minutes by a starch–iodide spray.

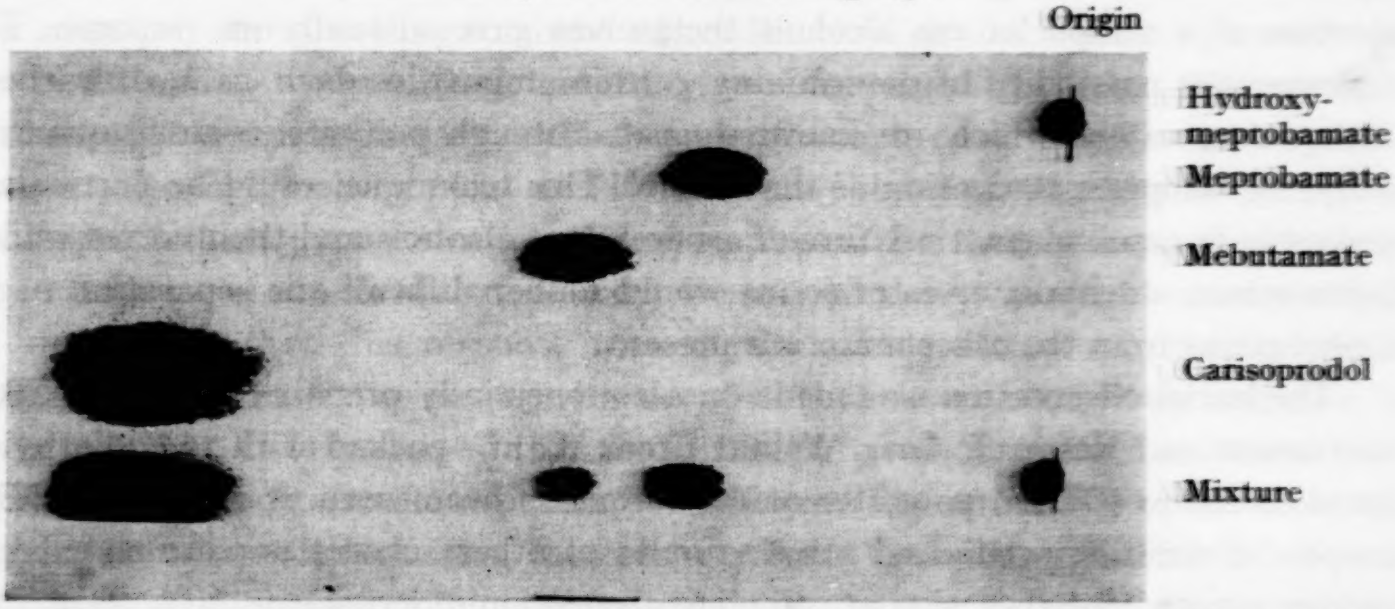

Fig. 1. Paper chromatogram of several important carbamates, 10 µg quantities. Solvent system is carbon tetrachloride–acetic acid–water (1:2:1, v/v/v).

The results, Fig. 1, indicate a good separation of the compounds studied and provide the following R_F values:

Carisoprodol	0.85
Mebutamate	0.45
Meprobamate	0.35
Hydroxymeprobamate	0.02

Wallace Laboratories
Division of Carter Products, Inc.,
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Received June 12th, 1961

J. Chromatog., 6 (1961) 540–541

Absorption of alcohols in gas–liquid chromatography applied to the determination of non-alcoholic impurities

The direct analysis of high molecular weight alcohols on polyester gas–liquid chromatography substrates has been reported to result in partial or complete absorption[1,2]. Long-chain fatty alcohols have therefore been separated on polyglycol substrates[3], non polar substrates[2,3,4], or on polyester substrates after acetylation of the alcohols[2,5,6].

While carrying out an analysis of acetylated long-chain fatty alcohols, prepared from marine oils, on a polyester column at high temperatures (220°), inadvertent injection of a sample of the alcohols themselves gave virtually no response. This suggested the possibility of determining certain impurities such as hydrocarbons, aldehydes or esters, which are known to pass through polyester columns quantitatively, by complete absorption of the alcohol. This technique would be particularly applicable in cases where a mixture of a number of alcohols and their corresponding hydrocarbon, aldehyde, or ester forms would render difficult the separation of the alcohol peaks from the other materials present.

The initial observation was made on a commercially prepared column (Wilkins Instrument and Research, Inc., Walnut Creek, Calif.) packed with 20 % diethylene glycol succinate (DEGS) polyester on 60–80 mesh Chromosorb. A number of DEGS samples of differing origin and some samples of other substrates were tested with varying results. However, similar complete absorption of long-chain fatty alcohols at high temperatures was observed with a DEGS polyester synthesized without catalyst (courtesy of DeSoto Chemical Coatings, Inc., Chicago, Ill.), and one prepared from diethylene glycol adipate (LAC-2R-446, Cambridge Industries Co., Inc., Cambridge, Mass.), containing 2% phosphoric acid. In the latter case the absorption activity was short-lived, whereas with the other two materials up to one hundred analyses of alcohols or of methyl esters were carried out before the column ceased to absorb quantitatively.

TABLE I

AREA RESPONSE, RELATIVE TO METHYL MYRISTATE AS 1.00, OF 80% OLEYL ALCOHOL MIXED WITH 20% METHYL MYRISTATE

Temp. °C	*Nature of column and sample size* *20% DeSoto DEGS 0.005 ml*	 *25% LAC-2R-446 with 2% H_3PO_4 0.020 ml*
180	1.55	0.10*
200	1.16	0.10*
220	0.61	0.08
240	0.03	0.03

* Approximate owing to extreme peak width.

To assess the sensitivity of the technique, and to reduce variations in instrument response due to temperature and carrier gas flow changes, samples of alcohol mixed with a pure methyl ester were employed. The area of the methyl ester peak was taken as 1.00 and the relative area of the alcohol peak was expressed in proportion. The results of tests at varying temperatures on two substrates are presented in Table I, where a mixture of 80% oleyl alcohol (prepared by reduction of highly purified methyl oleate with lithium aluminum hydride[6]) and 20% methyl myristate was employed.

The actual relative response shown in the presumed alcohol peak position under conditions believed to give total alcohol absorption varied from 3 to 6% of the methyl ester response, corresponding to about 1% of the oleyl alcohol. This is believed to be an actual impurity in the alcohol, and this view is supported by the fact that carefully crystallized palmityl and stearyl alcohols under the same conditions gave no response. However, the high melting point and limited solubility of these materials prevented equally high alcohol concentrations being employed. Further evidence that the observed response of the oleyl alcohol under these conditions was due to an impurity is the observation that this response was relatively independent of retention time, whereas preliminary experiments with decyl alcohol, never completely absorbed in any experiment, clearly showed that the amount of alcohol absorbed increased with retention time under fixed temperature conditions. The possibility of the observed response being an artifact from the column cannot be entirely discounted, but in preliminary studies trace amounts of material with the same retention time as methyl oleate were observed in preparations of oleyl alcohol other than the one employed in determining the results listed in Table I.

The sharp transition in column activity, when exhausted, from complete absorption to decreasing partial absorption suggests that the absorption of the alcohol takes place at a limited number of reactive groups in the polyester, and not at the ester linkages. While free carboxyl groups are a distinct possibility as active sites[1], it is also possible that carboxylic acid anhydrides would be present and would be very reactive. The authors suggest that specific polyesters might be produced to identify the active groups and then modified accordingly to increase the absorptivity toward long-chain fatty alcohols.

Experimental

The apparatus employed was a Wilkins Aerograph Model A-110-C. All columns were $^1/_4$-inch in diameter, and 10 feet in length. The filament current was maintained at 200 mA in all experiments. The flow rate of the carrier gas, helium, was varied to keep the retention times (for the methyl myristate) relatively constant at about 10 min at all temperatures.

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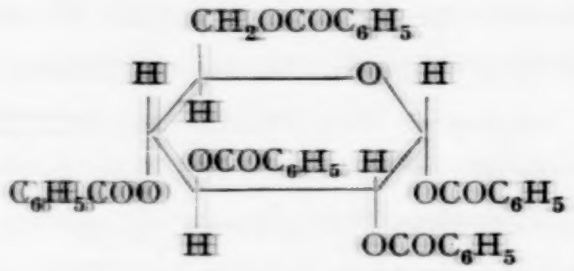
Received June 19th, 1961

The use of pentabenzoyl-α-glucose as stationary phase in gas chromatography

RECENTLY, MACZEK AND PHILLIPS[1] have used tri-o-thymotide (dissolved in tritolyl phosphate) as column liquid in gas chromatography, taking advantage of its well known ability to form crystalline inclusion compounds with different molecules. The particular structure of pentabenzoyl-α-glucose (PBG) suggested to us that this compound might be able to occlude some organic molecules. In another work[2], the absorption ability of PBG for some alcohols has been shown and the absorption isotherms and kinetics have been studied.



In the present note, the possibility of using PBG as selective stationary phase in gas–liquid chromatography is considered. Its high melting point permits its use at high temperatures without danger of decomposition, while its optical activity suggests its application for resolving optical antipodes. This latter possibility is now under investigation.

Preparation of the stationary phase. PBG was prepared according to the method of LEVENE AND MEYER[3] from D-α-glucose and benzoyl chloride, and purified by several crystallizations from ethyl alcohol containing 10% of pyridine and then from ethyl alcohol alone. M.p. 187°, $[\alpha]_D^{20°} = +138°$.

A mixed stationary phase of PBG and tricresyl phosphate (TCP) was prepared as follows: 10 g of PBG were dissolved in 50 ml of acetone and then 15 g of TCP were added. The clear solution was added to 50 g of Celite C22 80–120 mesh in a beaker and the mixture was allowed to evaporate in a water bath with continuous stirring. After complete evaporation of the solvent, the Celite support was dried in an oven at 100°. Two columns of 2 meter each, with 6 mm diameter, were used. As blank, 4 m columns containing TCP alone supported on Celite C22 were employed.

Apparatus. A gas chromatographic apparatus "FRACTOVAP" (Carlo Erba, Milan) was used with a Speedomax recorder (2.5 mV F.S.D.) and a detection system based on thermal conductivity (thermistors); 50 mA total bridge current.

The experimental conditions were:

column temperature	150°
inlet pressure	0.65 atm
gas flow rate	8 l/h
carrier gas	hydrogen
chart speed	0.5 in./min
sample size	5–10 μl
bridge current	28 mA

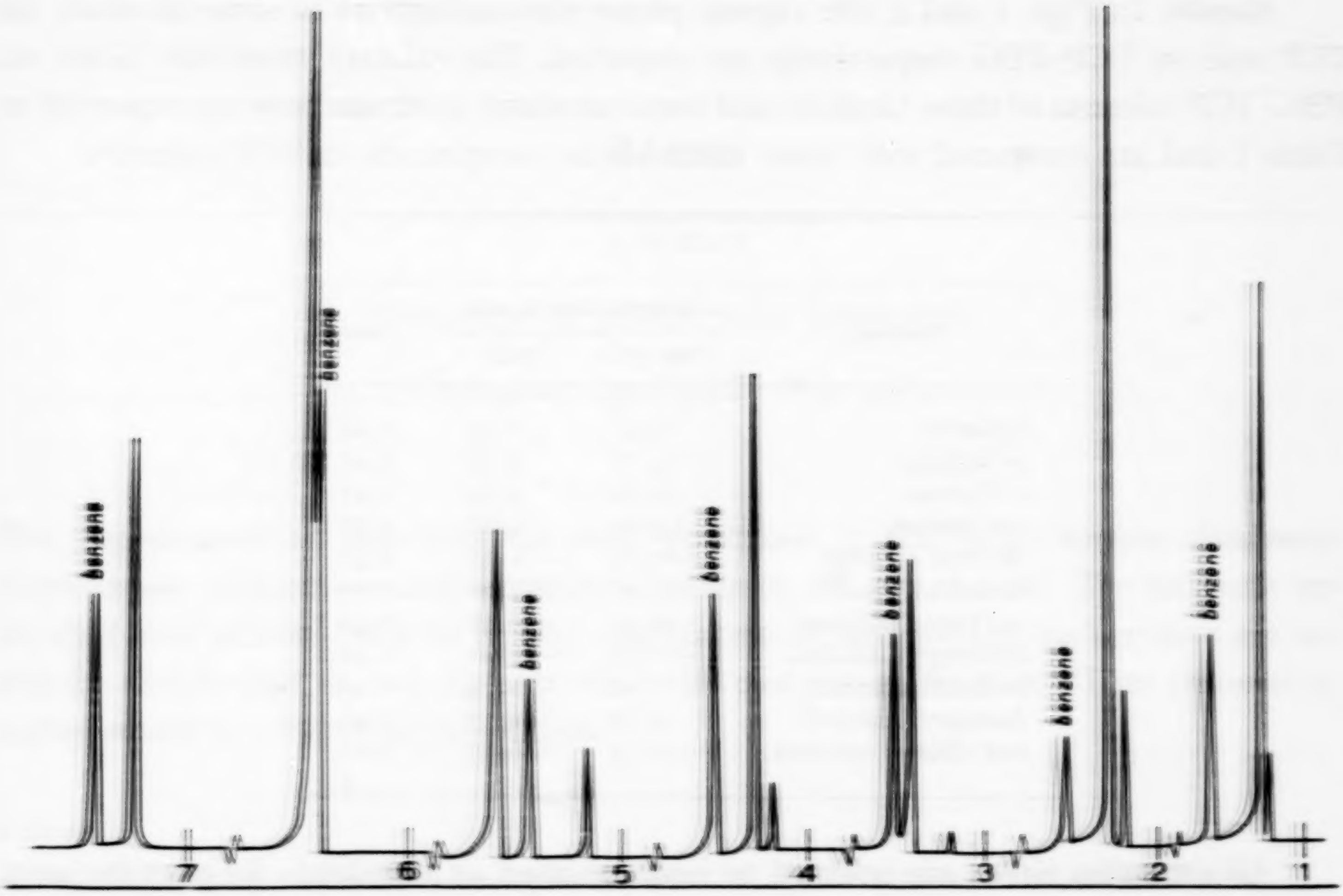


Fig. 1. Vapour phase chromatograms of some alcohols. Stationary phase: tricresyl phosphate 20% on Celite C22, 80–120 mesh; column length: 4 m; column temp.: 150°; carrier gas: hydrogen; gas flow rate: 8 l/h; inlet pressure: 0.65 atm; bridge current: 28 mA; chart speed: 0.5 in./min; sample size: 5–10 µl. (1) Methyl alcohol; (2) ethyl alcohol; (3) *n*-propyl alcohol; (4) isopropyl alcohol; (5) *n*-butyl alcohol; (6) isobutyl alcohol; (7) *tert.*-butyl alcohol.

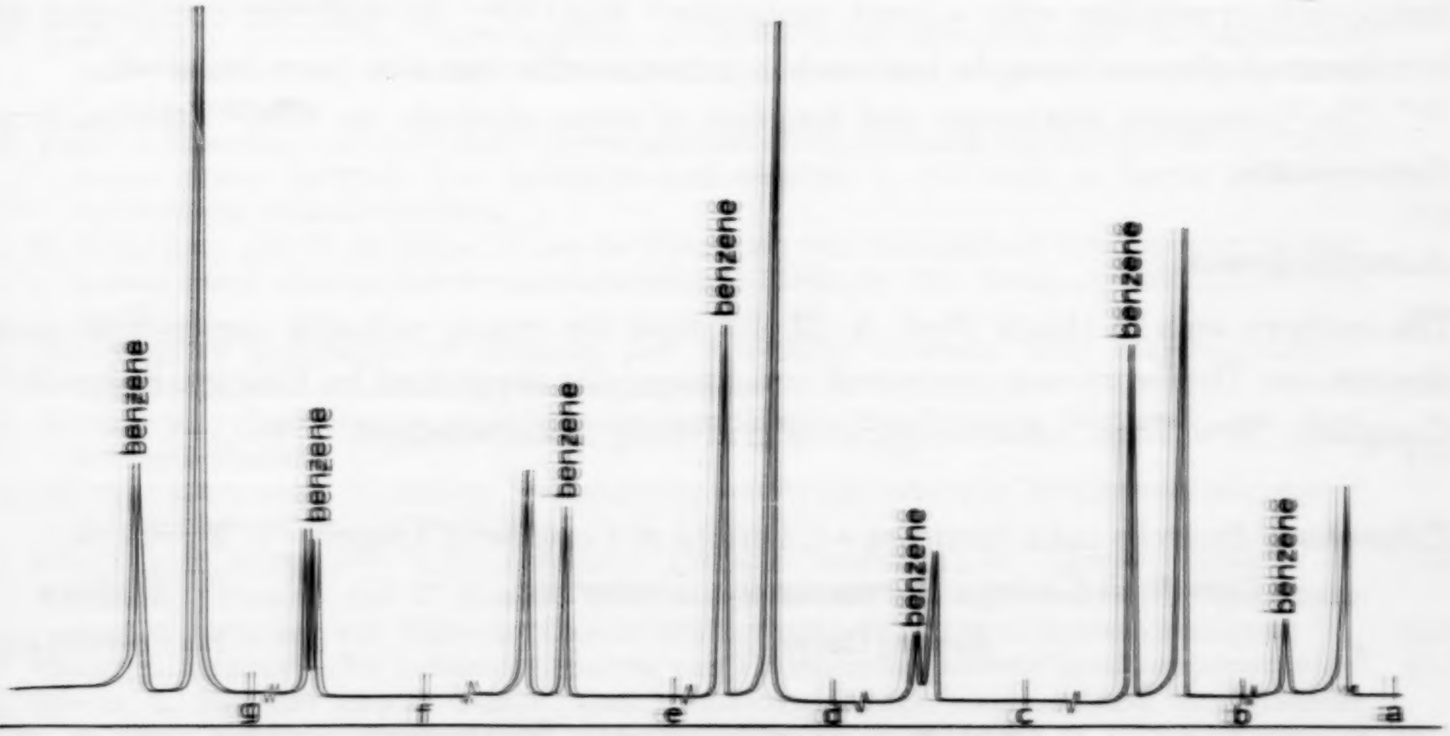


Fig. 2. Vapour phase chromatograms of some alcohols. Stationary phase: tricresyl phosphate 20% and pentabenzoyl-α-glucose 13% on Celite C22, 80–120 mesh; column length: 4 m; column temp.: 150°; carrier gas: hydrogen; gas flow rate: 8 l/h; inlet pressure: 0.65 atm; bridge current: 28 mA; chart speed: 0.5 in./min; sample size: 5–10 µl. (a) Methyl alcohol; (b) ethyl alcohol; (c) *n*-propyl alcohol; (d) isopropyl alcohol; (e) *n*-butyl alcohol; (f) isobutyl alcohol; (g) *tert.*-butyl alcohol.

Results. In Figs. 1 and 2, the vapour phase chromatograms of some alcohols on TCP and on TCP–PBG respectively are reported. The relative retention times on PBG–TCP columns of these alcohols and some aromatic hydrocarbons are reported in Table I and are compared with these of the same compounds on TCP columns.

TABLE I

Substance	*Retention time in min*		*Ratio*
	PBG–TCP	*TCP*	
Toluene	1.91	1.33	1.44
p-Xylene	4.71	3.33	1.41
o-Xylene	6.99	4.75	1.47
p-Cymene	13.66	9.66	1.38
Methyl alcohol	—1.74	—1.25	1.40
Ethyl alcohol	—1.50	—1.00	1.50
n-Propyl alcohol	—0.54	—0.37	1.46
Isopropyl alcohol	—1.33	—0.91	1.46
n-Butyl alcohol	1.12	0.75	1.49
Isobutyl alcohol	0.25	0.17	1.47
tert.-Butyl alcohol	—1.75	—0.87	2.01

All retention times are referred to benzene used as standard. As may be seen, these substances are retarded and hence selectively adsorbed with respect to TCP alone, when a solution of PBG in TCP is used as stationary phase. These results are in agreement with the hypothesis that holes may be formed in the lattice of PBG, in which organic molecules are occluded depending on their molecular shape.

Some evidence for this behaviour may be found in the older literature in which the preparation of glucose benzoates is reported. In fact, the authors claim that these compounds crystallize with solvent molecules[3]. Recently, an addition compound of tetrabenzoyl-glucose bromide and carbon tetrachloride has also been isolated[4].

The absorption isotherms and kinetics of some alcohols on PBG[2] also confirm these results.

Acknowledgements

The authors wish to thank Prof. A. M. LIQUORI for many valuable suggestions and discussions. This work was sponsored and financially supported by Colgate-Palmolive Company, New York, which support is gratefully acknowledged.

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[1] O. MACZEK AND C. S. G. PHILLIPS, *Gas Chromatography*, Butterworths, London, 1960, p. 284.
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Received May 24th, 1961

News

CONGRESS OF ANALYTICAL CHEMISTRY

Budapest, April 24th–29th, 1961

The papers read at this congress were published in the *Acta Chimica Academiae Scientiarum Hungaricae* and constitute volumes 26, 27 and 28. The subjects are divided into sections such as theory, application of isotopes etc. As the *Acta* are not widely distributed, it was thought that the contents of Section VI on chromatography would be of interest to readers:

Volume 27

J. INCZÉDY: Redox reactions on ion exchange columns. II. Reduction and oxidation of some inorganic and organic substances for analytical purposes 185
E. VÁMOS AND GY. MAJOR: Papierthermodiffusion 193
N. BRENNER AND L. S. ETTRE: Characteristics of capillary gas chromatography and its application to quantitative analysis 205
K. GÄRTNER, L. EBERT AND E. TAUBIG: Trennungsmöglichkeiten für Arsen, Molybdän und Wolfram durch Ionenaustauscher und Ionenaustauschermembranen 215
J. GASPARIČ: Papierchromatographie auf imprägnierten Papieren 221
G. GNAUCK: Probleme der Edelgasanalytik 229
M. HOFMANN AND H. G. STRUPPE: Ein Beitrag zur gas-chromatographischen Analyse von höheren Alkoholen und Paraffinen 239
Á. JÁSZ AND T. LENGYEL: Investigations on ion-exchange equilibria with the radioactive tracer method. II. Investigations concerning the removal factor in binary monovalent cationic systems 247
Á. JÁSZ, T. LENGYEL AND G. SCHAY: Investigations on ion exchange equilibria with the radioactive tracer method. III. Investigations relating to the removal factor in binary multivalent cationic systems 253
I. B. KELEMEN AND P. SZARVAS: Über die Trennung von Metallionen verschiedener Wertigkeiten durch eine papierchromatographische Methode. Die Trennung des Ti(III)s von Ti(IV) 261
I. KRAWCZYK: Separation of uranium and rare-earth elements from ethylenediaminetetraacetic acid solutions on ion-exchange resin 269
J. MLODECKA: Quantitative papierchromatographische Bestimmung der zwei- und dreiwertigen Phenole 279
A. OSZTROVSZKY AND J. UJSZÁSZ: Bestimmung von Fruchtestern im Branntwein mit papierchromatographischem Verfahren 285
I. RUSZNÁK, F. PÉTER AND A. GY. GERGELY: Einfache Verfolgung der Stärkehydrolyse 295
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P. SIMON, L. SZEPESY AND J. SIMON: Analyse von Flüssigkeiten mittels Gaschromatographie . 311
J. SIMON, L. SZEPESY AND P. SIMON: Gaschromatographische Untersuchung verschiedener flüssiger Kohlenwasserstoffe und sauerstoffhältiger Verbindungen 321
E. VÁMOS AND F. SIMON: Adsorptionschromatographische Methode zur Untersuchung von Alkylpolymethacrylaten 335
E. VÁMOS AND F. SIMON: Anwendung der Austausch-Chromatographie zur Bestimmung der Fettsäuren in Schmierfetten und Seifen 347

CONFERENCE ON PAPER CHROMATOGRAPHY OF THE CHROMATOGRAPHY GROUP OF THE CZECHOSLOVAK CHEMICAL SOCIETY AFFILIATED TO THE CZECHOSLOVAK ACADEMY OF SCIENCE

Prague, June 21st and 22nd, and Liblice, June 23rd, 1961

The chromatography group of the Czechoslovak Chemical Society organised a two-day conference on paper chromatography in Prague, at which original papers were read, and a one-day symposium on "the relationship between chromatographic behaviour and chemical structure of compounds" and on "systematic analysis" in Liblice.

While the papers read at the conference will be published in scientific journals, the discussion and communications at the symposium in Liblice will be published by the Czechoslovak Academy of Science as a separate volume.

The programme lists the following papers:

Opening Session

R. **Consden**: On the origin, development and future of paper chromatography.
V. V. **Račinskij**: Paper chromatography in the light of the general theory.
I. M. **Hais**: Remarks on some general problems of paper chromatography.

A. Chromatography of Inorganic Substances

F. H. **Pollard**: Advances in paper chromatography of inorganic substances (a review).
M. **Lederer**: Adsorption chromatography of inorganic substances on cellulose.
E. **Blasius**: Possible secondary reactions during the paper chromatographic separation of oxygen-containing acids of sulphur.
E. **Malý**: An attempt at paper chromatographic semiquantitative microdetermination of silica in the presence of phosphates, carbonates, sulphates and arsenates.
M. **Šteffek**: Analysis of cations by means of paper chromatography.

B. Advances in the Technique of Paper Chromatography

K. **Macek**: Introductory review.
A. J. P. **Martin**, J. B. **Jepson** and I. **Smith** (*film*): Principles of chromatography.
V. **Holeyšovský**, V. **Tomášek**, K. **Kochloefl** and K. **Setínek** (*film*): Technique of paper chromatography of amino acids and peptides.
B. **Keil**, J. **Hrdina** and B. **Meloun**: Automatic analysis of the course of ion-exchange chromatography by means of paper chromatography.
A. **Grüne**: New experiences with special papers for chromatography and ion-exchangers on cellulose basis.
C. S. **Knight**: Techniques of ion-exchange paper chromatography.
M. **Pavlíček**, Z. **Deyl** and J. **Rosmus**: Pressureless apparatus with central point development for centrifugal chromatography.
Z. **Deyl** and J. **Rosmus**: Adsorption chromatography in the centrifugal field. Chromatographic separation of gelatin fractions and of some amino acids.
J. **Rosmus** and Z. **Deyl**: Partition chromatography in the centrifugal field. Chromatography of the 2,4-dinitrophenylhydrazones of carbonyl compounds.
*A. **Waksmundski**: Determination of suitable solvent systems for counter-current distribution from paper chromatographic data.
Z. **Stránský**: A note on the detection in short-wave U.V. light.
O. **Horešovský**, Z. **Franc** and I. M. **Hais**: Frame for simultaneous processing of autoradiograms and other technical modifications.
J. **Fellegi** and L. **Slama**: Principles of reflectance photometry of paper chromatograms.

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* The authors marked with an asterisk did not participate at the conference.

C. Applications in Some Groups of Organic Compounds

J. Gasparič: Introductory review.
J. Borecký: Separation and identification of alcohols as monoalkyl sulphates.
V. Vorobjov: Identification of hydroxymethylated phenols.
K. Macek and S. Vaněček: Paper chromatography of some α,β,γ-substituted crotonolactones and some aromatic acids.
J. Franc: Identification of aromatic nitriles.
J. Gasparič and A. Cee: Paper chromatography of organo-tin compounds.
J. Latinák: Paper chromatography of naphthalenic intermediates in the production of dyes.
J. Latinák: Chromatographic separation of naphthylamine sulphonic acids in *n*-butanol–hydrochloric acid–water.
J. Gasparič and I. Táborská: Paper chromatography of dispersion dyes.
J. Šrámek: Paper chromatography of polycyclic and indigoid vat dyes.

D. Applications for the Analysis of Drugs and Substances of Natural Origin

J. Opieńska-Blauth: The losses of tryptophan during chromatographic analysis.
J. Opieńska-Blauth: Chromatographic methods in studies of amino-aciduria.
V. Jiráček, J. Koštíř and B. Jirešíková: Estimation of free and bound amino acids in plant material by means of paper chromatography.
L. Vámos: Chromatographic separation of sugar mixtures in biological fluids.
V. Betina: Paper chromatographic determination of the ionic properties of unknown antibiotics and of the conditions for their extraction.
Z. Šesták: Paper chromatography of chloroplast pigments (a review).
M. Kutáček, M. Valenta and J. Nováková: Paper chromatography of indolic compounds.
J. Ergala, J. Holub and J. Knap: Evaluation of the anthraquinones of cortex frangulae.
V. Bumba and S. Kudrnáč: Chromatographic separation of the substances from *Ammi majus*.
G. B. Marini-Bettòlo: Paper chromatographic analysis of *Strychnos* alkaloids.
D. Waldi: Systematic analysis of alkaloids.
*F. M. Šemjakin: Paper chromatography of sulfonamide preparations.
J. Večerková, M. Šulcová and K. Kácl: Chromatographic study of antihistamines and ataractics in reversed-phase systems.
J. Beštová: Application of paper chromatography for the determination of the reaction course of the methylation of aminoantipyrine and its derivatives.

E. Applications in the Chemistry and Biochemistry of Lipids

C. Michalec: Introductory review.
V. Koman: Application of paper chromatography for the systematic study of steric and position isomers of unsaturated fatty acids.
J. Pokorný: Separation of fatty acids by means of their urea addition compounds.
J. Churáček and J. Gasparič: Paper chromatography of fatty acids.
D. Halama and Č. Michalec: Quantitative paper chromatography of esters and glycerides.
V. Krajčiová, I. Čižnár and V. Krčméry: Chromatographic determination of fatty acids in the endotoxin of *Salmonella cholerae suis*.
M. Prusíková: A contribution to the chromatography of steroids.
J. Strašek and L. Stárka: Czechoslovak chromatographic paper S-100 and its application for the separation of lipophilic substances.
H. Fürst and K. Praeger: Paper chromatography of 1,2,3,4,5,6-hexachlorocyclohexane isomers.
K. Černý: Paper chromatography of some carotenoids.

The following contributed to the symposia at Liblice:

I. M. Hais, C. S. Knight, W. Matthias, J. Duchoň, G. B. Marini-Bettòlo, V. Vorobjov, J. Franc, E. R. Reichl, Ž. Procházka, K. Macek, J. Večerková, V. V. Račinskij, D. Waldi, V. Betina, F. H. Pollard (read *in absentia* by I. Smith), J. Šrámek, M. Lederer, D. Halama, M. Pöhm and V. Drozen.

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* The authors marked with an asterisk did not participate at the conference.

INTERNATIONAL SYMPOSIUM ON MICROCHEMICAL TECHNIQUES

The Pennsylvania State University, August 13th–18th, 1961

At this symposium the following papers on chromatography were read:

G. GRASSINI AND G. ALBERTI (Italy): A very sensitive fluorescence test for uranium; its use in the paper chromatographic estimation of uranium in natural waters.

C. MARESH, R. A. HOFSTADER, G. E. GERHARDT AND O. E. SUNDBERG (U.S.A.): Application of gas chromatography to the micro determination of carbon, hydrogen and nitrogen.

C. HISHTA AND R. F. RESCHKE (U.S.A.): Determination of micro quantities of chloroform in pharmaceutical products by gas chromatography.

E. G. WOLLISH (U.S.A.): Present status of thin-layer chromatography.

H. K. MANGOLD, R. KAMMERECK AND D. C. MALINS (U.S.A.): Thin-layer chromatography as an analytical and preparative tool in lipid chemistry.

L. S. ETTRE AND W. AVERILL (U.S.A.): Some recent developments in gas chromatography with respect to the analysis of extremely small quantities.

L. WEISNER AND W. J. SCHMIDT-KÜSTER (Germany): Performance characteristics of an integrating counter-detector for gas chromatography.

B. BOBRANSKI AND L. SYPER (Poland): Identification of drugs containing unsaturated radicals by paper chromatography.

S. PATTON (U.S.A.): The use of 2,4-dinitrophenylhydrazine in problems of flavor chemistry.

BOOK REVIEW

Partition of Cell Particles and Macromolecules, by PER-ÅKE ALBERTSSON, with a foreword by A. TISELIUS, Almqvist and Wiksell, Stockholm, 1960, 231 pages, price Sw.kr. 35.00.

This book is a thesis submitted for the degree of Doctor of Philosophy at the University of Uppsala, and as is usual only part of it has appeared previously in scientific journals. It thus presents, as mentioned in the foreword, the most complete and up-to-date description of the methods developed by the author.

Briefly these methods are the study of partition systems that have two water-rich phases, the measurement of the partition coefficients of whole cells, microsomes, bacteriophages, viruses as well as proteins and, finally, the application of these data to counter-current extraction and to the concentration and purification of viruses.

The partition systems consist of such mixtures as dextran–polyethylene glycol–water, dextran–methylcellulose–water or inorganic salt (*e.g.* magnesium sulphate)–polyethylene glycol–water. Pages 38 to 91 are mainly phase diagrams for such systems, the second, main, chapter (pages 95–159) deals with the distribution of the cell particles and macromolecules between these phase systems and again gives a wealth of numerical data, while the last two chapters deal with separations (Craig-machine) and concentration procedures.

We wholeheartedly agree with the publishers that the book is of interest to those working in the fields of biochemistry, cell physiology, microbiology, immunology, surface chemistry and colloid chemistry. It is well printed and bound and no printing errors were noted.

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CHROMATOGRAPHIC DATA

Vol. 6 (1961)

CHROMATOGRAPHIC DATA

SUPPLEMENT TO THE
JOURNAL OF CHROMATOGRAPHY
VOL. 6 (1961)

EDITORS:

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ELSEVIER PUBLISHING COMPANY
AMSTERDAM

PRINTED IN THE NETHERLANDS BY
DRUKKERIJ MEIJER N.V., WORMERVEER AND AMSTERDAM

EDITORIAL

COOPERATION IN DATA COLLECTION

R_F values and electrophoretic mobilities in paper have established themselves as new physical constants used in the characterisation of compounds. Their value, whether precise or approximate, is appreciated; their function in the comparison and contrast of similar compounds is widely recognised. They have the benefit of versatility by virtue of the changes that can be rung in solvent and electrolyte and are backed by the microanalytical spot test which, almost without fail, follows the separation. The first major clues to chemical identity are now very often obtained with the aid of the new physical constants. Their value in comparison, contrast and clue-seeking led to the inception of the present data section as an aid to research workers.

We now wish to enlist the cooperation of others, so that we may expand the present facilities, since the rapid increase in volume of work and publication precludes extensive coverage. To this end we ask authors to help (a) by sending regularly reprints of their papers containing R_F and electrophoretic mobility values (paper or thin solid layer) to:

Dr. C. B. Coulson,
c/o Arthur D. Little Research Institute,
Inveresk,
Midlothian,
Scotland

and (b) by giving in their paper all the essential details of method (solvent system and electrolyte composition, paper type, temperature of run, and mode of detection) as well as by placing all their values together.

TABLE 1

R_F VALUES OF SOME POLYAMINES

(U. Bachrach, S. Persky and S. Razin, *Biochem. J.*, 76 (1960) 306)

Solvent: Butanol–acetic acid–water (50:25:25).
Paper: Whatman No. 1 (K. V. Giri, A. N. Radhakrishnan and C. S. Vaidyanathan, *Nature*, 170 (1952) 1025).
Detection: 0.2% ninhydrin in butanol (spray).

Compound	R_F
Spermidine	0.13
Propane-1,3-diamine	0.24
β-Alanine	0.50
γ-Aminobutyric acid	0.58

TABLE 2

R_F VALUES OF SOME GLYCEROPHOSPHORIC ESTERS

(M. G. Macfarlane, *Biochem. J.*, 78 (1961) 44)

Solvents: S_1 = Phenol saturated with aqueous 1% NH_4OH.
S_2 = *tert.*-Butanol–water (62:38, w/v), trichloroacetic acid (10%, w/v).
Paper and detection: See R. M. C. Dawson, *Biochim. Biophys. Acta*, 14 (1954) 374.

Compound	R_F	
	S_1	S_2
Glycerophosphorylinositol	0.09	0.26
Polyglycerophosphate from cardiolipin	0.13	0.45
Glycerophosphorylserine	0.20	0.41
Glycerophosphate	0.25	0.61
Glycerophosphorylethanolamine	0.67	0.41

TABLE 3

R_F VALUES OF SOME GLYCEROPHOSPHORIC ESTERS

(C. Bradbeer and P. K. Stumpf, *J. Lipid Research*, 1 (1960) 214)

Solvents: S_1 = Butanol–propionic acid–water (40:30:7)
S_2 = Phenol saturated with water.
Paper: Whatman No. 1, acid washed.
Detection: Method of C. S. Hanes and F. A. Isherwood, *Nature*, 164 (1949) 1107, as modified by R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, 193 (1951) 405.

Compound	R_F*	
	S_1	S_2
Glycerophosphate	0.17	0.26
Glycerylphosphorylethanolamine	0.19	0.57
Glycerylphosphorylcholine	0.23	0.83
Glycerylphosphorylinositol**	0.08	0.10
Glycerylphosphorylglycerol**	0.13	0.38

* Obtained from two-dimensional chromatogram.
** Tentative identification.

TABLE 4

R_F VALUES OF RIBITOL, ITS DERIVATIVES AND CERTAIN OTHER COMPOUNDS

(J. J. ARMSTRONG, J. BADDILEY AND J. G. BUCHANAN, *Biochem. J.*, 76 (1960) 610)

Solvents: S_1 = Propanol–NH_4OH, sp. gr. 0.88–water (6:3:1).
S_2 = Butanol–ethanol–water–NH_4OH, sp. gr. 0.88 (40:10:49:1), organic phase.
Paper: Whatman No. 1 or No. 4 (not specified) (previously washed with 2 *N* acetic acid then water).
Detection: Improved periodate–Schiff reagent* (for α-glycols); molybdate reagent (for phosphoric esters); aniline phthalate (for reducing sugars); ninhydrin reagent (for amino acids).

Compounds	R_F	$R_{ribitol}$**
	S_1	S_2
Anhydroribitol	0.75	1.38
Glycerol	0.75	1.47
Ribitol	0.65	1.00
Glucose	0.61	0.65
Alanine	0.63	0.57
Glucosylribitol	0.55	0.55
Ribitol 1-phosphate	0.30	—
Ribitol 2(and 3)-phosphate	0.33	—
Anhydroribitol 5-phosphate	0.29	—
Glucosylribitol 1(and 2)-phosphate	0.28	—
Ribitol diphosphate	0.12	—
Glucosylribitol diphosphate	0.12	—
Glycerol diphosphate	0.12	—

[*] *Cf.* J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818.

[**] $R_{ribitol}$ = R_F compound/R_F ribitol.

TABLE 5

R_F VALUES (RELATIVE) OF TETROSES AND TETRITOLS

(R. D. BATT, F. DICKENS AND D. H. WILLIAMSON, *Biochem. J.*, 77 (1960) 272)

Solvents: S_1 = Butan-2-one–acetic acid–aq.4% boric acid (9:1:1).
S_2 = Ethyl acetate–pyridine–water (12:5:4).
Paper: Whatman No. 1 (descending).
Time of run: 16 h.
Detection: Aniline–phosphoric acid–acetic acid solution (*cf.* F. DICKENS AND D. H. WILLIAMSON, *Biochem. J.*, 64 (1956) 567); periodic acid–benzidine reagent (H. T. GORDON, W. THORNBURG AND L. N. WERUM, *Anal. Chem.*, 28 (1956) 849) for polyols and sugars having adjacent hydroxyls; thin end-window Panex monitor (model 50A) for ^{14}C.

Compound	R_G*	
	S_1	S_2
L-Erythrulose	8.9	2.25
D-Erythrose	10.1	2.00
Erythritol	5.9	1.56
D-Threitol	6.0	1.44

[*] R_G = R_F of compound/R_F glucose.

TABLE 6

R_F VALUES OF STRUCTURAL ISOMERS OF GLUTAMYLCYSTINYLVALINE

(D. MORRIS, *Biochem. J.*, 76 (1960) 349)

Solvents: S_1 = Butanol–acetic acid–water (63:10:27).
S_2 = Amyl alcohol–pyridine–water (7:7:6).
S_3 = Phenol–water (5:2)/NH_3 in tank.
Paper: Whatman No. 1 (descending).
Detection: Not given.

Compound	R_F		
	S_1	S_2	S_3
α-Peptide (disulphide form)	0.16	—	0.21
γ-Peptide (disulphide form)	0.12	—	0.42
α-Peptide (–SO_3H form)	—	0.43	0.17
γ-Peptide (–SO_3H form)	—	0.48	0.16

TABLE 7

ELECTROPHORETIC MOBILITIES OF STRUCTURAL ISOMERS OF GLUTAMYLCYSTINYLVALINE

(D. MORRIS, *Biochem. J.*, 76 (1960) 349)

Electrolyte: E_1 = 2 *N* acetic acid (pH 2.1).
E_2 = 0.35 *M* pyridine–acetic acid (pH 3.5).
Paper: Whatman No. 3 MM.
Apparatus: A_1 = uncooled low voltage.
A_2 = cooled high voltage.
Potential: P_1 = 25 V/cm (26 mA).
P_2 = 19 V/cm (14 mA).
P_3 = 66 V/cm (24 mA).
Units: cm.
Time of run: T_1 = 4 h.
T_2 = 1 h.
T_3 = 20 min.
Detection: Not given.

Compound	*Mobility*		
	$E_1P_1T_1$	$E_2P_2T_2$	$E_2P_3T_3$
α-Peptide (–SO_3H form)	3.0	9.0	2.3
γ-Peptide (–SO_3H form)	3.0	13.0	5.6

TABLE 8

R_F VALUES OF BENZOIC ACID AND SOME DERIVATIVES

(B. C. BALDWIN, D. ROBINSON AND R. T. WILLIAMS, *Biochem. J.*, 76 (1960) 595)

Solvents: S_1 = Butanol–acetic acid–water (4:1:5).
S_2 = Butanol–benzene–water (5:2:2).
S_3 = Butanol–NH_4OH soln. sp. gr. 0.88–water (4:1:5).
S_4 = Methyl ethyl ketone–2 *N* NH_4OH (1:1).
S_5 = Propanol–NH_4OH soln. sp. gr. 0.88 (7:3).
S_6 = Benzene–acetic acid–water (1:1:2).
(All ratios by vol.).

Paper: Whatman No. 4 (descending).

Time of run: 5–6 h (S_1, S_2, S_3, S_5); 2–2.5 h (S_4, S_6).

Length of run: 30 cm (approx.).

Detection: D_1 = Ninhydrin (1% in ethanol); heat to 100–120° for 10 min.
D_2 = *p*-Dimethylaminobenzaldehyde (1% in acetic anhydride); heat at 120° for 5 min.
D_3 = *p*-Dimethylaminocinnamaldehyde (0.05% in acetic anhydride); heat at 120–140° for 2 min.
D_4 = U.V. light (quenching at 270 mμ).

Compound	R_F						*Colour**			
	S_1	S_2	S_3	S_4	S_5	S_6	D_1	D_2	D_3	D_4
Benzoic acid	1.0	0.95	0.5	0.1	0.65	0.9	—	—	—	+
Benzoyl glucuronide**	0.7	0.15	0.25	0.05–0.1	0.35	0.0	—	—	—	+
Ornithuric acid	0.9–1.0	0.7	0.65	0.4	0.8–0.95	0.3	—	—	w (ybd)	+
Hippuric acid	0.85	0.2	0.3–0.4	0.1–0.2	0.05	0.1	—	o	r	+
N^5-Benzoylornithine	0.5	0.1	0.35	0.01***	0.6	0.0	p	—	w (ybd)	+
N^2-Benzoylornithine	0.46	0.1	0.27	0.01***	0.6	0.0	p	—	w (ybd)	+

---

* p = purple; o = orange; r = red; w (ybd) = white spot on yellow background. + indicates positive result, — negative result.

** Detected by naphtharesorcinol spray (see B. C. BALDWIN, D. ROBINSON AND R. T. WILLIAMS, *Biochem. J.*, 71 (1959) 638).

*** Can be separated by 10 h run.

TABLE 9

R_F VALUES OF DIHYDROXYPHTHALIC ACIDS AND PROTOCATECHUIC ACID

(D. W. RIBBONS AND W. C. EVANS, *Biochem. J.*, 76 (1960) 310)

Solvent: Butan-1-ol–acetic acid–water (40:10:50, upper phase).

Paper: Whatman No. 4.

Detection: Diazotised *p*-nitroaniline or diazotised sulphanilic acid followed by Na_2CO_3 (H. G. BRAY AND W. V. THORPE, *Methods of Biochem. Anal.*, 1 (1954) 27); Folin-Ciocalteau reagent then exposure to NH_3 fumes (F. L. MITCHELL AND R. E. DAVIES, *Biochem. J.*, 56 (1954) 690); molybdate–nitrite reagent (C. B. COULSON AND W. C. EVANS, *J. Chromatog.*, 1 (1958) 374); U.V. light.

Compound	R_F
3,4-Dihydroxyphthalic acid	0.52
4,5-Dihydroxyphthalic acid	0.49
Protocatechuic acid	0.73

TABLE 10

R_F VALUES OF SOME CONDENSED TANNINS

(D. G. ROUX AND E. PAULUS, *Biochem. J.*, 77 (1960) 315)

Solvents: S_1 = 2% aq. acetic acid (ascending).
S_2 = Butan-2-ol saturated with water.
S_3 = Butan-1-ol–acetic acid–water (4:1:5).
Paper: Whatman No. 3.
Detection: Toluene-*p*-sulphonic acid (D. G. ROUX, *Nature*, 180 (1957) 973).

Compound	R_F		
	S_1	S_2	S_3
(+)-Fustin	0.37*; 0.43	0.81	0.80
(+)-Mollisacacidin	0.55; 0.57		
(—)-Robinetinidol	0.42*		
(+)-Catechin	0.36*		
(—)-Fustin	0.35*	0.81	0.80
(±)-Fustin	0.36*	0.81	0.80
(—)-7, 3′, 4′-Trihydroxyflavan-3,4-diol	0.49	0.76	

* Determined on the same chromatogram.

TABLE 11

R_F VALUES OF SOME FLAVONOID COMPOUNDS

(D. G. ROUX AND E. PAULUS, *Biochem. J.*, 78 (1961) 120)

Solvents: S_1 = 2% aqueous acetic acid.
S_2 = Butan-2-ol saturated with water.
S_3 = Butan-1-ol–acetic acid–water (4:1:5).
Paper: P_1 = Whatman No. 1 (not specified).
P_2 = Whatman No. 3 (ascending).
Detection: Ammoniacal silver nitrate; bis-diazotised benzidine; ferric alum; toluene-*p*-sulphonic acid.

Compound	R_F			
	S_1P_1	S_1P_2	S_2P_1	S_3P_1
(+)-Fustin	0.37	0.43	—	—
(—)-Mollisacacidin	—	0.57	—	—
(—)-Fisetinidol	0.48	—	0.82	0.81
(+)-Fisetinidol	0.43	—	0.82	0.81

TABLE 12

R_F VALUES OF SOME DEOXYRIBOSIDES AND RELATED COMPOUNDS

(D. W. A. ROBERTS, Research Station, Lethbridge, Alberta, Canada, unpublished results)

Solvents: S_1 = *n*-Propanol–tetrahydrofurfuryl alcohol–water–0.75 *M* citrate pH 5.7 (20:10:9:1 by vol.).
S_2 = *n*-Propanol–ammonium hydroxide (sp. gr. 0.90)–water (3:1:1 by vol.).
S_3 = Isobutyric acid–acetic acid–water (100:1:50 by vol.).
S_4 = *n*-Butanol–acetic acid–water (5:4:1 by vol.).
S_5 = Isobutyric acid–ammonium hydroxide (sp. gr. 0.90)–water (60:1:33 by vol.).
S_6 = *tert.*-Butyl alcohol–water–formic acid (sp. gr. 1.22) (16:4:1 by vol.).
Paper: Whatman No. 1 (descending).
Time: 20 h.
Detection: (1) Ultraviolet from Mineralight Model SL 2537.
(2) Dische reagent (0.5% cysteine hydrochloride in 3 *N* H_2SO_4) followed by heating at 85° (approx. 5 min). This reagent does not give satisfactory results after irrigation with S_2.

Compound	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
Adenine	0.57	0.64	0.83	0.64	0.90	0.43
Cytosine	0.50	0.52	0.57	0.52	0.65	0.43
Guanine	0.40	0.35	0.46	0.19	0.55	0.23
Hypoxanthine	0.53	0.52	0.54	0.47	0.55	0.37
Thymine	0.71	0.71	0.70	0.63	0.71	0.66
Uracil	0.72	0.55	0.54	0.51	0.62	0.72
Xanthine	0.54	0.41	0.34	0.21	0.42	0.28
Adenosine	0.54	0.62	0.75	0.48	0.74	0.37
Inosine	0.46	0.49	0.42	0.33	0.43	0.29
Uridine	0.69	0.50	0.38	0.40	0.43	0.52
Deoxyadenosine	0.64	0.68	0.83	0.60	0.86	0.46
Deoxycytidine	0.59	0.57	0.53	0.55	0.61	0.42
5-Methyl-deoxycytidine	0.65	0.68	0.57	—	0.64	0.42
Deoxyguanosine	0.61	0.53	0.53	0.26	0.54	0.35
Deoxyinosine	0.54	0.56	0.49	0.45	0.53	0.37
Deoxyuridine	0.62	0.61	0.51	0.54	0.59	0.56
Thymidine	0.78	0.75	0.64	0.60	0.65	0.65
Deoxyribose	0.73	0.64	0.46	0.56	0.48	0.57

TABLE 13

R_F VALUES OF SOME URIDINE DERIVATIVES

(J. SMRT, J. BERÁNEK AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2459)

Solvent: Isopropanol–ammonia–water (7:1:2).
Paper: Whatman No. 1.
Detection: Not given.

Compound	R_F
Uridine-5'-O-acetate-3'-phosphate	0.22
2'-O-Tetrahydropyranyl-uridine-5'-O-acetate-3'-phosphate	0.38
6-Azauridylyl-(5' → 3')-uridine	0.24

TABLE 14

R_F VALUES OF SOME FLAVINS

(I. G. WHITE AND G. J. LINCOLN, *Biochem. J.*, 76 (1960) 301)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, upper phase).
S_2 = 5% (w/v) Na_2HPO_4.
S_3 = Methanol.
S_4 = Phenol–butan-1-ol–water (160:30:100, w/v/v).
Paper: Whatman No. 1 (descending, S_1, S_2, S_4; ascending, S_3).
Detection: U.V. light.

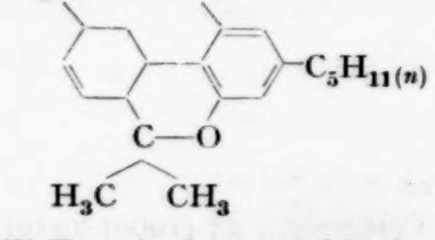
Compound	R_F			
	S_1	S_2	S_3	S_4
Riboflavin	0.25	0.30	0.16	0.80
Riboflavin phosphate	0.04	0.56	—	0.15
Lumichrome	0.65	0.05	0.28	—
Flavinadenine dinucleotide	0.03	0.41	0.00	0.17
Lumiflavin	0.41	0.15	—	—

TABLE 15

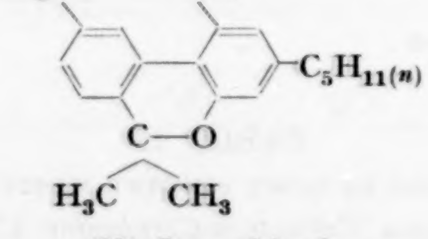
R_F VALUES OF SOME HASHISH CONSTITUENTS

(F. KORTE AND H. SIEPER, *Angew. Chem.*, 72 (1960) 210)

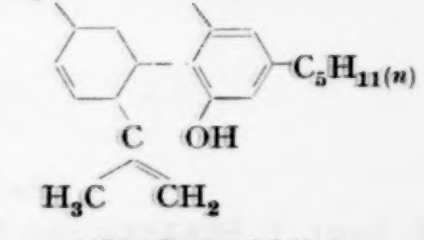
Solvent: Ligroin–benzene–chloroform–methanol–water (2:2:1:4:1, aqueous phase).
Paper: Schleicher & Schüll 2043a hy (hydrophobic).
Detection: D_1 = Beam reagent (W. BEAM, *cf. Chem. Zentr.*, (1924 II) 1255) followed by Pauly reagent (F. G. FISCHER AND H. DÖRFEL, *Z. physiol. Chem.*, 297 (1954) 278).
D_2 = Beam reagent followed by 2,6-dichloroquinone chloroimide (E. HOFFMAN AND G. HOFFMAN, *Naturwiss.*, 45 (1958) 337).
D_3 = Beam reagent followed by diazotised *p*-nitroaniline (A. GRÜNE, *Chimia (Switz.)*, 11 (1957) 173, 213).
D_4 = 2,6-Dichloroquinone chloroimide followed by diazotised *p*-nitroaniline.
D_5 = 2,6-Dichloroquinone chloroimide followed by Pauly reagent.
D_6 = U.V. absorption on chromatogram (λ max; mμ) (F. KORTE AND H. SIEPER, *Angew. Chem.*, 70 (1958) 434; 71 (1959) 455).



(I) Tetrahydrocannabinol



(II) Cannabinol



(III) Cannabidiol

Compound	R_F	Detection*					
		D_1	D_2	D_3	D_4	D_5	D_6
I	0.53	ly	cb	ly	ly	cb	274–280
II	0.67	oc-bn	b-gn	o	oc-bn	b-gn	284
III	0.74	di-dy	li	r-bn	o	li	274–280

* b = blue; bn = brown; cb = cobalt blue; d = dark; di = dirty; gn = green; l = lemon; li = lilac; o = orange; oc = ochre; r = red; y = yellow.

TABLE 16

R_F VALUES OF ORGANO-TIN COMPOUNDS

(D. J. WILLIAMS AND J. W. PRICE, *Analyst*, 85 (1960) 579)

Paper: Whatman No. 1.
Temperature: 24 ± 1°.
Solvents: (a) Pyridine–water (60:40).
(b) Butanol–pyridine–water (7.5 : 3.5, saturated with water).
(c) Butanol–ammonia–water (*N* in ammonia and saturated with water).
(d) Butanol–ethanol–water (3 : 1, saturated with water).

Compound	R_F			
	a	*b*	*c*	*d*
Me_2SnCl_2	0.36 T	0.55 T	0.03	0.67 T
Me_3SnCl	0.35	0.25	—	0.32
$EtSnCl_3$	streaks in all solvents			
Et_2SnCl_2	0.36 T	0.80 T	0.16	0.98 T
Et_2SnOAc	0.40	0.85 T	—	0.95 T
Et_3SnOH	0.88	0.94	0.94	0.95
Pr_2SnCl_2	0.00 T	0.9 T	streaks to 0.52	0.98
$(Pr_3Sn)_2O$	0.85	0.94	—	—
$(isoPr_3Sn)_2O$	0.87	0.94	—	—
$Pr_3SnCH_2CH_2COONa$	0.82	0.90	0.83	—
$BuSnCl_3$	streaks to 0.8	0.99 T	streaks length of paper	
Bu_2SnCl_2	0.00 T	0.93	0.00 T	0.98 slight tail
Bu_2Sn dilaurate	0.00 T	0.95	—	0.96
Bu_3SnCl	0.85	0.92	0.95	0.94
Bu_3Sn laurate	0.82	0.92	—	0.94
$(Bu_3Sn)_2O$	0.82	0.97	—	0.96
Bu_3Sn abietate	0.84	0.97	0.94	—
Hexabutyl distannane	—	0.94 (0.8, 0.3)	0.95 (0.8, 0.3)	—
Bu_4Sn	0.00	0.93	0.94	0.92
n-Hexyl$_2$Sn dilaurate	0.00	0.95	—	0.95
n-Octyl$_2$Sn dilaurate	0.00	—	—	0.94
n-Octyl$_3$SnCl	0.00	0.94	0.93	0.95
n-Octyl$_4$Sn	0.00	0.90	0.94	0.83
$PhSnCl_3$	streaks to 0.85	0.99 T	streaks length of paper	
Ph_2SnCl_2	0.00	0.00	0.00	0.95 T
Ph_3SnCl	0.85	0.95	0.97	0.97
Ph_3SnOAc	0.85	0.95	0.97	0.97
Ph_4Sn	0.00	0.00	0.00	0.00
$SnCl_4$	0.00	0.00	0.00	0.00

T = tailing.

TABLE 17

R_F VALUES OF ADDITION COMPOUNDS OF OLEFINS WITH MERCURIC ACETATE

(W. HUBER, *Mikrochim. Acta*, (1960) 44)

Solvent: Butanol mixed with 1.5 N NH_3 + 1.5 N $(NH_4)_2CO_3$ (1:1) (two phase).

Paper: Schleicher & Schüll 2043b (paper first dipped in aqueous phase, then blotted; final chromatographic development with organic phase) (descending).

Preparation of addition compound: Olefin mixture (gas or liquid) dissolved in pure methanol (0.5 g/100 ml), is shaken 2 h at room temperature with an excess of solid mercuric acetate (10–20 µl of supernatant taken). Hg acetate remains on start.

Detection: $(NH_4)_2S$ or dithizone spray after 5 min exposure to HCl fumes.

Olefin	R_F	*Olefin*	R_F
Ethylene	0.10	4-Methylpent-2-ene*	0.50/0.60
Propylene	0.15	2-Methylpent-1-ene	0.62
Isobutylene	0.22	*n*-Hex-2-ene*	0.59/0.63
But-1-ene	0.28	*n*-Hex-1-ene	0.70
2-Methylbut-2-ene	0.32	2,3-Dimethylpent-2-ene	0.70
2-Methylbut-1-ene	0.36	Isooctene	0.80
n-Pent-2-ene*	0.38/0.43	Cyclooctene	0.76
n-Pent-1-ene	0.50	Cyclopentadiene	0.17
Cyclohexene	0.46	Dicyclopentadiene	0.83
2-Methylpent-2-ene	0.48	Styrol	0.56

* These olefins yield two spots of equal intensity.

TABLE 18

R_F VALUES OF ADDITION COMPOUNDS OF OLEFINS WITH MERCURIC ACETATE

(W. HUBER, *Mikrochim. Acta*, (1960) 44)

Solvent: Propanol just saturated with 1.5 N NH_3–1.5 N $(NH_4)_2CO_3$ (1:1).

Paper: Schleicher & Schüll 2043b (descending).

Preparation of addition compound: Olefin mixture (gas or liquid) dissolved in pure methanol (0.5 g/100 ml) is shaken 30 min at room temperature with excess solid mercuric acetate (10–20 µl of the supernatant taken). Hg acetate remains at start.

Detection: $(NH_4)_2S$ or dithizone spray after 5 min exposure to HCl fumes.

Olefin	R_F*
Ethylene	0.47
Propylene	0.54
Isobutylene	0.62
2-Methylbut-1-ene	0.68
2-Methylbut-2-ene	0.68
Cyclohexene	0.72
n-Hex-1-ene	0.79
Isooctene	0.84
Isoprene	0.09/0.12/(0.53)/(0.68)
1,5-Hexadiene	0.14/(0.73)
Dipentene	0.15/0.31**

* R_F values in brackets are for weaker spots—perhaps impurities.

** Impure?

TABLE 19

R_F VALUES OF ADDITION COMPOUNDS OF OLEFINS WITH MERCURIC ACETATE

(W. HUBER, *Mikrochim. Acta,* (1960) 44)

Solvent: 1.5 N NH_3 + 1.5 N $(NH_4)_2SO_3$ (1:1) mixed with isoamyl alcohol (two phases). Aqueous phase used.

Paper: Schleicher & Schüll 2043b (impregnated with organic phase of solvent) (descending).

Preparation of addition compound: Olefin mixture (gas or liquid) dissolved in pure methanol (~ 0.5 g/100 ml) is shaken 2 h at room temperature with an excess of solid mercuric acetate (10–20 µl of supernatant taken). Hg acetate runs near front.

Detection: $(NH_4)_2S$ or dithizone spray after 5 min exposure to HCl fumes.

Olefin	R_F	*Olefin*	R_F
Hg acetate	0.82	Propylene tetramer	0.05
Ethylene	0.74	Cetene	0.05
Isobutylene	0.56	Propylene trimer*	0.7–0.4**
Diisobutylene	0.38	Propylene tetramer*	0.17
Triisobutylene	0.05	Triisobutylene*	0.13
Dodecene	0.05		

* Determined on a chromatogram with front run off. R_F of isooctene found to be 1.00.

** Tailing.

TABLE 20

R_F VALUES OF SOME COUPLING PRODUCTS OF p-NITROBENZENEDIAZONIUM SULPHATE WITH ACETONE

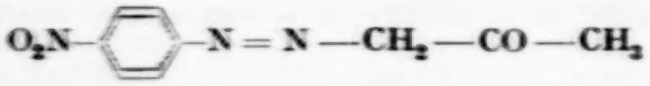
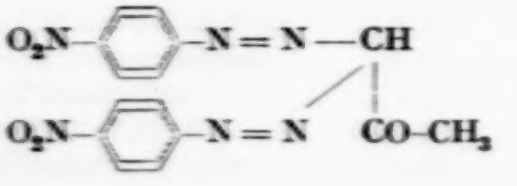
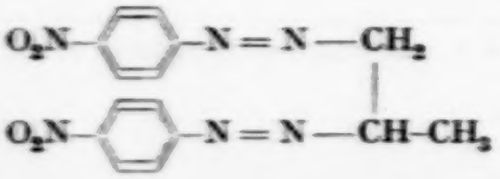
(Z. J. ALLAN AND J. PODSTATA, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1324)

Solvent: Methanol–butanol–water (80:5:15).

Paper: Whatman No. 1.

Impregnation: Liquid paraffin–ethanol (1:19) (30 min drying to remove ethanol followed by spotting of substances in pyridine solution and another 20 min drying).

Detection: 2.5 N NaOH spray followed by exposure to dioxane fumes; visible light.

Compound	*Colour*		R_F
	Actual	*50% NaOH*	
	weak yellow	intense red	0.85
1,3-Bis-(p-nitrobenzeneazo)-triazene	yellow	reddish violet	0.65
	orange	blue*	0.55
Unknown	orange	weak blue	0.30
	orange	greenish blue	0.15
Decomposition product of diazonium salt	orange	orange**	0.00

* Colour intensified by dioxane.

** Dioxane yields conversion to yellow-brown.

TABLE 21

R_F VALUES OF SOME DYESTUFF FISSION PRODUCTS

(J. Panchartek, Z. J. Allan and F. Mužík, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2783)

Solvents: S_1 = *n*-Butanol saturated with 2.5 *N* HCl.
S_2 = 2.5 *N* HCl.

Paper: Whatman No. 1 (a piece of fused sodium sulphide was added in certain cases* to produce H_2S (a) to complex copper in paper and (b) to provide a reducing atmosphere). Descending.

Detection: Paper exposed to nitrous gases for diazotization then sprayed with sodium 2-naphthol-3,6-disulphonate or resorcinol solution and finally made alkaline in an ammonia atmosphere; U.V. fluorescence.

Compound	R_F		*U.V.*	*Colour***			
	S_1	S_2		C_1	C_2	C_3	C_4
p-Phenylenediamine	0.07	0.82		gv	rg	blgn	g
m-Phenylenediamine	0.09	0.89	blgn	rb	yb		yb
7-Amino-8-hydroxyquinoline-5-sulphonic acid	0.09	0.74			y	wr	
5-Amino-8-hydroxyquinoline	0.10	0.80	ygn	g	rg	r	wy
3,3'-Dihydroxybenzidine	0.12	0.34			gb	gbl	
2-Naphthylamine-4,8-disulphonic acid	0.13	0.54	bl	blsr	wy		
2,5-Diamino-4-methoxytoluene	0.20	0.90		rv	b	blgn	
Benzidine	0.31	0.62	ygn	b	rg	wggn	
Aniline-*p*-sulphonic acid	0.34	0.93	wgn	o	wy		
Aniline-*m*-sulphonic acid	0.34	0.92		o	wy		
2-Naphthylamine-7-sulphonic acid	0.44	0.67		blsr			wblsr
2,6-Diamino-1-naphthol-3-sulphonic acid	0.46	0.60	blv	v	b		
Aniline-*o*-sulphonic acid	0.54	0.88		o	wy		re
5-Aminosalicylic acid	0.64	0.69	blgn			gbl	
Pyrazolone derivative III	0.64	0.71		ygn			
m-Aminophenol	0.70	0.78	blgn	y	yb		yb
Aniline	0.84	0.85		r	gy		r
m-Chloroaniline	0.89	0.74		r***	gy		b
p-Chloroaniline	0.91	0.82		mr	rg		
2,5-Dichloroaniline-4-sulphonic acid	0.93	0.87†		yr	wy		
2,5-Dichloroaniline	0.97	0.84		yr			

---

* For 4-amino-1-phenyl-3-methylpyrazol-5-one and some of its derivatives.

** C_1 = diazotization and coupling with 2-naphthol-3,6-disulphonic acid; C_2 = 24 h oxidation in air; C_3 = indophenol test; C_4 = with *p*-nitrobenzenediazonium chloride; bl = blue; b = brown; g = grey; gn = green; r = red; bls = bluish; m = muddy; v = violet; o = orange; w = weak; y = yellow; re = rose.

*** Immediately red after diazotization.

† Elongated spot.

TABLE 22

R_F VALUES OF SOME ARYLAZO-β-NAPHTHOLS

(J. Gasparič, M. Novotná and M. Jureček, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2757)

Solvents: S_1 = Hexane.
S_2 = Ethanol–ammonia (4:1).
S_3 = Ethanol–water (4:1).
S_4 = 85% acetic acid.
S_5 = 90% acetic acid.
Papers: Whatman No. 3 or WF_{II} Papier (Papierfabrik Niederschlag) (both ascending).
Impregnation: I_1 = Ethanolic dimethylformamide.
I_2 = 10% liquid paraffin in hexane.
I_3 = 10% 1-bromonaphthalene in methanol.
Temperature of run: 18–22°.
Detection: Visible light.

Parent amine	R_F					
	S_1I_1	S_2I_2	S_3I_2	S_4I_2	S_5I_3	*Colour**
Aniline	0.51	0.42	0.54	0.60	0.26	o
o-Chloroaniline	0.49	0.41	0.51	0.56	0.21	o
m-Chloroaniline	0.55	0.32	0.45	0.53	0.20	o
p-Chloroaniline	0.56	0.35	0.48	0.53	0.20	or
2,5-Dichloroaniline	0.65	0.25	0.39	0.41	0.12	o
o-Bromoaniline	0.46	0.36	0.51	0.53	0.18	o
m-Bromoaniline	0.55	0.29	0.43	0.47	0.18	o
p-Bromoaniline	0.52	0.29	0.44	0.50	0.16	o
o-Iodoaniline	0.39	0.39	0.48	0.45	0.15	o
p-Iodoaniline	0.45	0.26	0.40	0.43	0.11	or
o-Toluidine	0.65	0.39	0.50	0.53	0.24	r
m-Toluidine	0.66	0.35	0.50	0.54	0.24	r
p-Toluidine	0.67	0.34	0.48	0.53	0.24	or
4-Chloro-2-aminotoluene	0.69	0.25	0.36	0.38	0.15	r
5-Chloro-2-aminotoluene	0.68	0.23	0.33	0.40	0.15	r
m-Nitroaniline	0.12	—	—	0.75	0.21	o
p-Nitroaniline	0.09	—	—	0.76	0.24	r
2,4-Dinitroaniline	0.02	—	—	0.79	0.17	or
p-Chloro-*o*-nitroaniline	0.14	—	—	0.71	0.16	r
o-Chloro-*p*-nitroaniline	streak	—	—	0.68	0.10	r
p-Nitro-*o*-toluidine	0.20	—	—	0.72	0.18	or
o-Nitro-*p*-toluidine	0.20	—	—	0.72	0.18	or
m-Nitro-*p*-toluidine	0.12	—	—	0.79	0.22	c
o-Xylidine	0.68	0.26	0.42	0.44	0.20	r
m-Xylidine	0.69	0.31	0.43	0.50	0.23	or
p-Xylidine	0.72	0.31	0.46	0.46	0.20	r
o-Anisidine	0.19	0.59	0.70	0.75	0.30	c
p-Anisidine	0.36	0.45	0.58	0.67	0.27	r
p-Chloro-*o*-anisidine	0.29	0.50	0.68	0.69	0.20	c
p-Nitro-*o*-anisidine	0.10	—	—	0.79	0.20	v
o-Phenetidine	0.41	0.54	0.68	0.69	0.23	c
p-Phenetidine	0.51	0.38	0.51	0.52	0.19	r
α-Naphthylamine	0.48	0.30	0.41	0.46	0.14	v
β-Naphthylamine	0.42	0.30	0.42	0.47	0.15	c
4-Aminodiphenyl	0.40	0.26	0.38	0.40	0.10	c

---

* o = orange; c = carmine; r = red; v = violet.

TABLE 23

R_F VALUES OF SOME NAPHTHALENESULPHONIC ACIDS

(J. LATINÁK, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1649)

Solvents: S_1 = 37% HCl–water (1:3).
S_2 = *n*-Propanol–ammonia (2:1).
Paper: P_1 = Schleicher & Schüll No. 2043a (S_1).
P_2 = Whatman No. 3 (S_2) (descending).
Length of run: 30–35 cm.
Time of run: 7 h(P_2).
Detection: 0.05% Pinacryptol Yellow, then U.V. viewing.

Acid	R_F		*Fluorescence**
	S_1P_1	S_2P_2	
Naphthalene-1-sulphonic	0.71	0.78	bo
Naphthalene-2-sulphonic	0.64	0.78	yo
Naphthalene-1,3-disulphonic	0.62	0.66	o
Naphthalene-1,5-disulphonic	0.86	0.46	y
Naphthalene-1,6-disulphonic	0.81	0.47	o**
Naphthalene-1,7-disulphonic	0.86	0.51	ro
Naphthalene-2,6-disulphonic	0.76	0.46	o
Naphthalene-2,7-disulphonic	0.76	0.46	o
Naphthalene-1,3,5-trisulphonic	0.96	0.27	y
Naphthalene-1,3,6-trisulphonic	0.88	0.24	o
Naphthalene-1,3,7-trisulphonic	0.96	0.27	y
Naphthalene-1,3,5,7-tetrasulphonic	0.98	0.15	o

* b = brown, y = yellow, o = orange, r = red.
** On drying fluorescence changes to red to violet-brown in colour.

TABLE 24

R_F VALUES OF SOME 2-NAPHTHOLMONOSULPHONIC ACIDS

(J. LATINÁK, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 403)

Solvents: S_1 = *n*-Butanol–acetic acid–water (4:1:5).
S_2 = *n*-Propanol–aqueous $NaHCO_3$ (2:1).
Paper: P_1 = Whatman No. 4 (descending).
P_2 = Whatman No. 1 (descending).
Impregnation: I_1 = No impregnation.
I_2 = Aqueous 5% $NaHCO_3$.
Length of run: 35 cm.
Detection: U.V. light in NH_3 fumes; diazotised *p*-nitroaniline before and after NH_3 or 1% NaOH treatment.

2-Naphthol-sulphonic acid	R_F			
	$S_1P_1I_1$	$S_1P_2I_1$	$S_2P_2I_1$	$S_2P_2I_2$
2,3-	0.68	0.54	0.76	0.85
2,4-	0.54	0.39	0.68	0.55
2,5-	0.47	0.34	0.66	0.60
2,6-	0.47	0.35	0.65	0.60
2,7-	0.48	0.36	0.65	0.65
2,8-	0.54	0.40	0.66	0.68
2,1-	0.71	0.57	0.75	0.95

TABLE 25

R_F VALUES OF SOME 2-NAPHTHOLSULPHONIC ACIDS

(J. Latinák, *Collection Czech. Chem. Commun.*, 26 (1961) 403)

Solvent: S_1 = *n*-Propanol–aqueous $NaHCO_3$ (2:1).
Paper: Whatman No. 4 (descending).
Impregnation: I_1 = No impregnation.
I_2 = 5% aqueous $NaHCO_3$.
Length of run: >35 cm.
Detection: D_1 = U.V. light fluorescence in NH_3 fumes.
D_2 = Diazotised *p*-nitroaniline, original colour.
D_3 = Diazotised *p*-nitroaniline, after NH_3 fume exposure or 1% NaOH spray.

2-Naphthol-sulphonic acid	R_F[a]				Colour[c]		
	S_1I_1	S_1I_2	S_1I_1[b]	S_1I_2[b]	D_1	D_2	D_3
2,3,6,8-	0.03	0.02	—	—	lg	or	or-v
2,1,3,6-	0.07	0.04	—	—	lnb	o[d]	rv[d]
2,4,8-	0.09	0.06	—	—	lnb	or	or
2,5,7-	0.13	0.11	0.08	0.11	lg	o	rv
2,6,8-	0.13	0.12	—	—	lb	yo	yo
2,3,6-	0.16	0.10	0.10	0.16	lb	ro	rar
2,3,7-	0.19	0.14	0.12	0.19	lg-gb	or	vb
2,3,8-	0.19	0.15	—	—	lg-gb	ro	ro
2,3,5-	0.23	0.15	0.17	0.25	lg-gb	or	rv
2,1,5-	0.30	0.45	—	—	bv	wro[d]	wo[d]
2,1,6-	0.30	0.45	—	—	rv	wro[d]	wor[d]
2,1,7-	0.33	0.51	—	—	v	wro[d]	wo[d]
2,3-	0.74	0.84	0.83	0.74	lb	rar	rar
2,4-	0.66	0.54	0.73	0.53	drv	rar	bv
2,5-	0.63	0.62	0.73	0.54	lb	vr[e]	rv
2,6-	0.63	0.61	0.69	0.53	dbv	ro	rv
2,7-	0.63	0.65	0.66	0.46	lvb	o	rv
2,8-	0.65	0.68	—	—	lb	yo	yo
2,1-	0.74	0.94	—	—	wdv	wor	wor
2-	0.90	0.97	—	—	dv	ro	ro

---

[a] Values for 2-naphthol-di- and -trisulphonic acids calculated from $R_{2,6}$ values ($R_{2,6}$ = R_F of sulphonic acid/R_F of the 2,6 acid).

[b] R_F values of iodo-derivatives.

[c] b = blue; d = dark; g = green; l = light; n = navy; o = orange; r = red; ra = raspberry; v = violet; w = weak; y = yellow.

[d] Spot only visible after longer time.

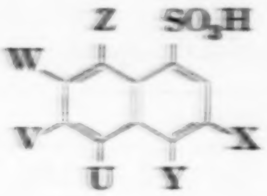
[e] Spot with orange edge.

TABLE 26

R_F VALUES (RELATIVE) OF SOME NITRONAPHTHALENESULPHONIC ACIDS

(J. HORYNA AND V. HANOUSEK, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 79)

Solvent: *n*-Butanol–ethanol–25% NH_4OH–water (60:20:4:16).
Paper: Whatman No. 1 (descending).
Impregnation: 2% $(NH_4)_2SO_4$ solution; only 4 cm wide strip at start.
Time of run: 50 h.
Detection: Sprayed with 20% aqueous hydrogen sulphite solution and developed in a stream of nitrogen (fluorescence in U.V. light).



I. U = SO_3H; X = NO_2*
II. U = SO_3H; Y = NO_2
III. V = SO_3H; Z = NO_2
IV. V = SO_3H; X = NO_2
V. U = NO_2
VI. U = SO_3H; X, Z = NO_2
VII. V = SO_3H; X, W = NO_2

Compound	R_Y**	*Colour****
I	0.24	bv
II	0.16	g
III	0.18	bg
IV	0.25	v
V	1.00	$g^{sh}b$
VI	0.20	$g^{sh}b$
VII	0.33	v

* Other letters = H; this applies to all formulae.

* R_Y = R_F compound/R_F compound V.

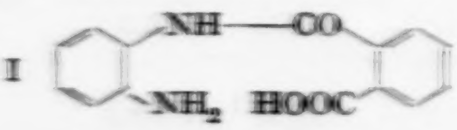
** b = blue; g = green; v = violet; g^{sh} = greenish.

TABLE 27

R_F VALUES OF SOME IMIDAZOLE DERIVATIVES

(J. ARIENT AND J. MARHAN, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 98)

Solvent: Cyclohexane–pyridine (3:1).
Paper: Whatman No. 1.
Detection: U.V. fluorescence; diazotisation with nitrous fumes, then coupling with alkaline R-salt solution.

Compound	R_F	*Colour of fluorescence**
2-(*o*-Carboxyphenyl)-benzimidazole	0.2	b
Intermediate I 	0.25	nil**
N-(*o*-Aminophenyl)-phthalimide·HCl	0.45	nil**
o-Di-(2-benzimidazolyl)-benzene	0.65	yg
1,2-Benzoylene-benzimidazole	0.8	y

* b = blue; yg = yellow-green; y = yellow.

** After diazotisation and then coupling with R-salt a red colour results.

TABLE 28

R_F VALUES OF SOME ORGANIC ACIDS AND THEIR DERIVATIVES

(H. F. MUELLER, T. E. LARSON AND M. FERRETTI, *Anal. Chem.*, 32 (1960) 687)

Solvents: S_1 = Ethanol–butan-1-ol–conc. NH_4OH (5:4:5).
S_2 = Butan-1-ol–acetic acid–water (4:1:5).
S_3 = Pentan-1-ol–5 *M* formic acid (1:1).
Paper: Whatman No. 1 (ascending).
Detection: 1% ethanolic $FeCl_3$, containing 0.1% HCl (hydroxamates); 0.04% bromophenol blue (in 95% ethanol at pH 6.7); 0.1% neutral ethanolic mercurochrome solution.

Acid	R_F		
	$S_1{}^a$	$S_2{}^b$	$S_3{}^c$
Butyric	0.37	0.78	—
Crotonic	0.31	0.78	—
Propionic	0.28	0.66	—
Acetic	0.16	0.58	—
Pyruvic	—	0.55	0.06, 0.62
Formic	0.14	0.48	—
Alkylbenzenesulphonic (ABS, an anionic detergent)	0.77	—	0.72, 0.04
Lactic	0.14	0.49	0.55
Succinic	—	0.55, 0.52, 0.47[d]	0.60
Gallic	0.09	—	0.48
Malonic	0.02	—	0.50
Oxalic	—	0.59, 0.55[d]	—
Aconitic	—	0.53, 0.66[d]	0.67
l-Malic	—	0.57, 0.52, 0.44, 0.49[d]	0.32
Citric	0.23	0.52	0.23
Isocitric	—	0.59, 0.45[d]	—
Tartaric	—	0.52	0.15

[a] Sodium salt.
[b] Hydroxamate.
[c] Acid.
[d] Hydroxamates not satisfactory. Two or three values for a single acid given in order of intensity.

TABLE 29

R_F VALUES OF SOME GUANIDINE BASES

(J. P. BASS, *Biochem. J.*, 77 (1960) 484)

Solvents: S_1 = Butan-1-ol–formic acid–water (7:1:2, by vol.).
S_2 = Propan-2-ol–acetic acid–water (15:1:4, by vol.).
Paper: Whatman No. 1 (descending).
Detection: Sakaguchi reagent, pentacyanoferriate reagent (I. SMITH, *Chromatographic Techniques*, William Heinemann Medical Books Ltd., London, 1958, p. 149).

Compound	R_F*	
	S_1	S_2
Arginine	0.07	0.17
γ-Guanidobutyric acid	0.45	0.56
Glycocyamine	0.23	0.38
Taurocyamine	0.11	0.29
Creatine	0.35	0.33

* Figures represent the mean of at least four determinations; S.D. is about 10%.

TABLE 30

R_F VALUES OF SOME PHENOLIC GLYCOSIDES

(J. D. ANDERSON, L. HOUGH AND J. B. PRIDHAM, *Biochem. J.*, 77 (1960) 564)

Solvents: S_1 = Butan-1-ol–pyridine–water (6:4:3, by vol.).
S_2 = Butan-1-ol–ethanol–water (40:11:19, by vol.).
S_3 = Ethyl acetate–acetic acid–water (9:2:2, by vol.).
Paper: Whatman No. 1 (descending).
Detection: Diazotised *p*-nitroaniline–Na_2CO_3 (T. SWAIN, *Biochem. J.*, 53 (1953) 200).

Compound	R_F		
	S_1	S_2	S_3
Arbutin	0.75	0.40	0.51
p-Hydroxyphenyl *β*-gentiobioside	0.55	0.07	0.17
p-Hydroxyphenyl *β*-cellobioside	0.61	0.10	0.24
Salicin	—	0.56	0.48
o-Hydroxybenzyl *β*-glucoside	—	0.59	0.48

TABLE 31

ELECTROPHORETIC MOBILITIES OF SOME PHENOLIC GLYCOSIDES

(J. D. ANDERSON, L. HOUGH AND J. B. PRIDHAM, *Biochem. J.*, 77 (1960) 564)

Electrolyte: 0.1 *M* sodium borate buffer (pH 10).
Paper: Whatman No. 3.
Apparatus: J. B. PRIDHAM (*J. Chromatog.*, 2 (1959) 605).
Units: M_{SA}, mobility relative to salicylic acid.
Detection: Diazotised *p*-nitroaniline–Na_2CO_3 (T. SWAIN, *Biochem. J.*, 53 (1953) 200).

Compound	M_{SA}
Arbutin	—
p-Hydroxyphenyl *β*-gentiobioside	0.28
p-Hydroxyphenyl *β*-cellobioside	0.22
Salicin	0.00
o-Hydroxybenzyl *β*-glucoside	0.15

TABLE 32

R_F VALUES OF SOME PHENOLIC COMPOUNDS

(W. J. BURKE, A. D. POTTER AND R. M. PARKHURST, *Anal. Chem.*, 32 (1960) 727)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Butan-1-ol–ethanol–formic acid–water (5:1:1:1).
S_3 = 15% aq. acetic acid.
S_4 = Chloroform–propan-2-ol–acetic acid–water (1:2:1:1).
S_5 = Butan-2-ol saturated with water.
Paper: Whatman No. 1 (ascending).
Temperature of run: 26.7° ± 1° (in solvent-saturated atmosphere).
Detection: Neutral silver nitrate (saturated)–acetone reagent (50 ml:1 l; sufficient water to dissolve precipitate), dip or spray.

Compound	$R_F \times 100$					*$AgNO_3$ colour**	*Reaction time** (h)*
	S_1	S_2	S_3	S_4	S_5		
Benzoic acids							
3-(OH)	90–95	92–96	68–74	90–94	92–95	lp	1–2
4-(OH)	90–95	88–93	68–77	86–92	92–95	p–t	1–2
2,4-(OH)$_2$	90–95	91–96	61–71	88–94	35–50	p	1–2
3,4-(OH)$_2$	73–81	85–92	64–70	74–80	94–95	db	2–3
2,3,4-(OH)$_3$	75–83	83–89	50–61	65–72	0–45	g	i
3,4,5-(OH)$_3$	61–68	63–70	56–61	55–60	—	b	i
4-(OH)-3-(OCH$_3$)	87–92	89–94	65–75	93–95	91–95	p–o	1
4-(OCH$_3$)	89–94	88–95	68–78	92–97	91–95	—	—
3,4-(OCH$_3$)$_2$	88–94	90–95	73–82	93–96	90–93	—	—
3,4-(OCH$_2$O)	89–94	91–95	63–73	93–96	91–95	lp	3–4
Cinnamic acids							
3-OH	89–93	90–95	60–70	89–93	94–96	p–t	3–4
4-(OH)	87–92	91–96	56–65	88–94	94–96	p–t	1–3
3-(OH)-4-(OCH$_3$)	86–91	90–96	46–56	85–90	94–97	lb	1–2
4-(OH)-3-(OCH$_3$)	86–93	90–95	50–63	91–95	94–97	b	3
3-(OCH$_3$)	90–94	90–95	64–74	90–97	94–96	—	—
4-(OCH$_3$)	89–94	89–95	50–63	92–96	94–97	—	—
3,4-(OCH$_3$)$_2$	90–95	93–96	54–64	95–97	95–97	—	—
3,4-(OCH$_2$O)	89–94	95–97	44–62	90–95	95–97	p–t	1–3
3,4,5-(OCH$_3$)$_3$	88–94	89–94	69–78	94–97	92–95	—	—
Phenols							
2-(OH)	88–92	90–95	75–83	88–94	94–97	gr	12–24
3-(OH)	90–94	89–93	73–79	90–94	95–97	b	2–3
4-(OH)	88–93	87–93	77–83	85–88	95–96	g–gr	1–2
2,3-(OH)$_2$	70–80	80–95	68–74	70–76	90–94	g	i
3,5-(OH)$_2$	72–83	73–77	64–71	61–66	89–94	r–b	1–3
2-(OH)-3-(OCH$_3$)	83–88	84–90	70–77	89–93	95–97	g	1–2
2,6-(OCH$_3$)$_2$	89–94	—	77–83	94–97	95–97	lb	1–2
Miscellaneous							
Rufigallic acid	0	0	0	0	0–05	t	i
Hexahydroxydiphenyl	47–57	45–55	47–54	38–47	0–40	r–b	i
Hexahydroxybenzophenone	70–75	65–77	31–36	47–53	—	y–b	0–1
m-Digallic acid	58–64	65–73	51–57	51–57	0–25	lt	0–2
Flavellagic acid	0	15–30	—	0–20	0–06	b	i
Ellagic acid	23–35	30–48	0–08	0–35	0–13	lt	0–1
Galloflavin	33–41	25–40	02–22	26–36	0–15	br–b	i
Isogalloflavin	—	27–45	0–23	27–34	0–07	b	i
β-Methyltropolone	90–96	78–95	81–97	93–97	94–96	lg	1–2
Purpurogallin	80–87	80–90	0–31	75–82	0–50	p–b	i
Purpurogallin-carboxylic acid	62–71	64–75	—	47–62	0–08	lt	2–3

* l = light; p = pink; t = tan; d = dark; b = brown; g = grey; o = orange; gr = green; r = red; br = brick.

** i = immediate.

TABLE 33

R_F VALUES OF SOME PHENOLS AND RELATED COMPOUNDS

(I. A. PEARL AND P. F. MCCOY, *Anal. Chem.*, 32 (1960) 1407)

Solvents: S_1 = Butanol saturated with 2% aqueous NH_4OH at 28°;
S_2 = Butanol–pyridine–water (10:3:3) at 28°;
Paper: Whatman No. 1 (descending);
Temperature of run: 28°;
Detection: D_1 = Fast Scarlet GR salt; Naphthanil Diazo Scarlet 2G (2,5-dichloroaniline);
D_2 = Fast Orange GG salt (*m*-dichloroaniline);
D_3 = Fast Blue VB salt (4-amino-4′-methoxy-diphenylamine);
D_4 = Fast Bordeaux GP Salt (2-amino-3-nitroanisole);
D_5 = Fast Black Salt K (4-amino-2,5-dimethoxy-4′-nitroazobenzene);
D_6 = Fast Bordeaux Salt BD (4-amino-2,5-dimethoxybenzonitrile);
D_7 = Fast Corinth Salt V (4-amino-2,4′-dimethyl-5-methoxy-2′-nitroazobenzene);
D_8 = Fast Red Salt GG (*p*-nitroaniline);
D_9 = Fast Red Salt RL (2-amino-3-nitrotoluene);
D_{10} = Fast Yellow Salt GC (*o*-chloroaniline);

(Note: These are commercial names of the stabilized diazo salt of the compound in parentheses). The solvent is allowed to evaporate, the spot exposed to NH_3 fumes and sprayed immediately with 0.05% aqueous diazo salt. The paper is then air dried, the colour noted, then sprayed with saturated aqueous Na_2CO_3 after 30 min and the colour again noted.

Compound	R_F		Colour*									
	S_1	S_2	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}
Acetosyringone	0.38	0.76	y–bn	y–bn	y–bn	y–bn	bn	y–bn	bn	y–bn	bn–gd	y–bn
Acetovanillone	0.60	0.83	p	s	p	p	v	l–ph	pu	pu	pu	o
Caffeic acid	0.03	0.34	bn	bn–gn	bn	d–gn	g–bn	bn–gn	bn	d–bn	bn	b
p-Coumaric acid	0.18	0.57	v	c	pu	bn–re	v	v	p	b–g	bn–pu	p
2,5-Dihydroxyacetophenone	0.85	0.91	y–gn	y–gn	y–gn	y–gn	y–bn	d–gn	y–bn	y–gn	y–gn	d–gn

2,5-Dihydroxyacetophenone	0.85	0.91	y–gn	y–gn	y–gn	y–gn	y–bn	d–gn	y–bn	y–gn	y–gn	d–gn
2,5-Dihydroxybenzoic acid	0.13	0.30	g–gn	bn	y–bn	g–gn	bn–gd	d–gn	y–bn	bn	bn	g–bn
3,5-Dihydroxybenzoic acid	0.06	0.44	gd	y	pu	d–o	b–v	o	pu	o	o	gd
Ferulic acid	0.13	0.48	pu	p	v	p	g–pu	b	b–g	g–gn	bn	pu
p-Hydroxybenzaldehyde	0.77	0.85	o	y–gd	o	l–t	bn	c	bn	re	bn–re	ph
p-Hydroxybenzoic acid	0.10	0.56	o	gd	o	o	v	c	bn–o	re	pu	gd
2-Hydroxy-5-methoxybenzoic acid	0.38	0.47	pu	re	l–bn	v	w**	bn–p	bn	w	bn–r	s
p-Hydroxyphenyl-acetic acid	0.09	0.62	r–o	c	p	re	bn	pu	b–g	v	pu	s
Protocatechuic acid	0.07	streak	bn	bn	l–bn	gn	bn	bn–gn	bn	bn–pu	bn–pu	bn
Quinic acid	0.01	0.05	—	—	—	—	—	—	—	—	—	—
Salicyl alcohol	0.84	0.86	p	gd	pu	y–o	b	v	b–v	o	pu	gd
Syringaldehyde	0.37	0.74	w	c	p	l–y	bn–oe	pu	bn–gn	b	y	g–gn
Syringic acid	0.05	0.31	c	o	pu	pu	b	v	b	b	v	s
Vanillic acid	0.10	0.41	o	o	r–bn	bn–o	g–b	v	b–v	v	pu	o
Vanillil	0.19	0.87	y–bn	y–gd	y–gd	y–gd	gd	bn–gd	y–bn	bn–gd	y	y
Vanillin	0.44	0.87	c	bn	p	p	g–b	v	b–v	v	pu	o

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[*] After satd. aqueous Na_2CO_3; b = blue; bn = brown; c = coral; d = dark; g = grey; gd = gold; gn = green; l = light; o = orange; oe = olive; p = pink; ph = peach; pu = purple; r = red; re = rose; s = salmon; t = tan; v = violet; w = white; y = yellow.

[**] Colour due to sizing and resultant spray rejection.

TABLE 34

R_F VALUES OF SOME HALOGENATED PHENOLS

(H. S. Choguill and D. E. Bissing, *Anal. Chem.*, 32 (1960) 440)

Solvents: S_1 = Benzene–acetic acid–water (2:2:1, by vol.).
S_2 = Hydrochloric acid (20%, w/w).
Paper: Whatman No. 1.
Temperature of run: 25°.
Detection: 1% *p*-Nitrobenzenediazonium fluoborate spray followed by 5% KOH in methanol spray (*cf.* J. H. Freeman, *Anal. Chem.*, 24 (1952) 958).

Compound	R_F range		
	S_1	S_2	*Colour**
o-Chlorophenol	0.67–0.70	0.72–0.83	a
p-Chlorophenol	0.07–0.16	0.70–0.83	a
o-Bromophenol	0.45–0.48	0.87–0.88	b
p-Bromophenol	0.31–0.32	0.80–0.82	b
o-Iodophenol	0.91–0.94	0.88–0.90	b
p-Iodophenol	0.90–0.94	0.54–0.57	b

* a = light red; b = dark red.

TABLE 35

R_F VALUES OF SOME QUINONES AND PHENOLS

(A. T. Diplock, J. Green, E. E. Edwin and J. Bunyan, *Biochem. J.*, 76 (1960) 563)

Solvents: S_1 = Cyclohexane–benzene*.
S_2 = Benzene.
Paper: Not given (60 cm wide).
Impregnation: $ZnCO_3$*.
Time of run: S_1, 1 h; S_2, not given.
Detection: U.V. light.

Compound	R_F	
	S_1	S_2
α-Tocopherol	0.71	
Ubiquinone 50	0.77	
RC-ubiquinone	0.73	
Ubiquinol 50	0.73	
o-Cresol		0.63
m-Cresol		0.44
p-Cresol		0.44
2-Methoxyphenol		0.26
3-Methoxyphenol		0.33
4-Methoxyphenol		0.33
γ-Tocopherol	0.45	

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* J. Bouman and E. C. Slater, *Biochim. Biophys. Acta*, 26 (1957) 624.

TABLE 36

R_F VALUES OF SOME AMINOPHENOLS AND DERIVATIVES

(D. V. PARKE, *Biochem. J.*, 77 (1960) 493)

Solvents: S_1 = Diisopropyl ether saturated with water (washed with satd. aq. $KMnO_4$, satd. aq. $FeSO_4$, aq. 2 *N* Na_2CO_3, then water; dried over Na_2SO_4; distilled, b.p. 68°).

S_2 = $CHCl_3$-formic acid–methanol–water (1000:4:100:96, by vol.), (L. REIO, *J. Chromatog.*, 1 (1958) 338).

S_3 = Benzene–methyl ethyl ketone–formic acid–water (900:100:2:98, by vol.), (L. REIO, *J. Chromatog.*, 1 (1958) 338).

S_4 = Ethanol–butan-1-ol–aqueous 3 *N* NH_4OH/3 *N* ammonium carbonate buffer (11:40:19, by vol.) (M. E. FEWSTER AND D. A. HALL, *Nature*, 168 (1951) 78).

Paper: Whatman No. 1 (descending).

Time of run: 6 h (S_1, S_3); 8 h (S_2); 16 h (S_4).

Detection: D_1 = Gibb's reagent (ethanolic 2% 2,6-dichloroquinone-chloroimide, then 2 *N* Na_2CO_3).

D_2 = Diazo reagent, acid (1% $NaNO_2$ in 0.5 *N* HCl followed by 1% ethanolic dimethyl-α-naphthylamine).

D_3 = D_2 followed by 2 *N* Na_2CO_3.

D_4 = aq. 0.5% $FeCl_3$ followed by saturated $NaHCO_3$ (pH values in parentheses).

D_5 = aq. 0.2% Brentamine Fast Blue 2B salt followed by aq. 2 *N* NH_4OH.

D_6 = aq. 0.2% Brentamine Fast Garnet GBC salt followed by aq. 2 *N* NH_4OH.

D_7 = 1% naphthoresorcinol in aq. 20% trichloroacetic acid followed by heating (100°, 20–30 min).

D_8 = aq. 0.1% $BaCl_2$ followed by aq. 0.1% rhodizonic acid (sodium salt).

Compound	R_F				Colour[*]							
	S_1	S_2	S_3	S_4	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8
o-Aminophenol	0.79	0.93	0.96	—	bl	y	gn	b	y	y		
m-Aminophenol	0.60	0.81	0.89	—	v	r	y	neg.	o	r		
p-Aminophenol	0.38	0.85	0.98	—	bl-b[**]	v	gn	neg.	v	v		
3-Aminocatechol	—	0.90	0.96	—	bl			v(7) v(9)	y	y		
4-Aminocatechol	—	0.33	0.46	—	bl			gn(7) v(9)	v	v		
2-Aminoresorcinol	—	0.87	0.87	—	v v			pi(7) bu(9)	r	r		
4-Aminoresorcinol	—	0.44	0.80	—	v			o(7) v(9)	v	r		
5-Aminoresorcinol	—	0.01	0.02	—	neg.			neg.	v	r		
Aminoquinol	—	—	—	—	neg.			neg.	neg.	neg.		
Aniline-N-glucuronide	—	—	—	0.09		v					bl[***]	neg.
p-Aminophenyl-glucuronide	—	—	—	0.02		b					b[***]	neg.
Glucuronic acid	—	—	—	0.02		neg.					bl[***]	neg.
Phenylsulphamic acid	—	—	—	0.26		v					neg.[***]	w
Acetanilide	—	—	—	0.83		v					neg.[***]	neg.
Aniline†	—	—	—	0.87		v					neg.[***]	neg.

---

[*] bl = blue; b = brown; gn = green; r = red; v = violet; y = yellow; o = orange; w = white on red background; pi = pink; bu = buff; neg. = negative.

[**] Also reported as negative.

[***] Detection reagent applied after hydrolysis by spraying with *N* HCl and heating (100° for 10 min).

† Volatile, therefore only amounts of 200 μg or more can be detected.

TABLE 37

R_F VALUES OF *m*-DINITROBENZENE AND ITS METABOLITES

(D. V. PARKE, *Biochem. J.*, 78 (1961) 262)

Solvents: S_1 = Benzene–acetic acid–water (1:1:2, by vol.).
S_2 = Diisopropyl ether saturated with water (D. V. PARKE, *Biochem. J.*, 77 (1960) 493).
Paper: Whatman No. 1 (descending).
Time of run: 6 h (S_1).
Detection: D_1 = 0.1 *N* NaOH.
D_2 = 1% aq. $FeCl_3$.
D_3 = Gibb's reagent (0.2% ethanolic 2,6-dichloroquinone-chloroimide followed by 2 *N* Na_2CO_3).
D_4 = 0.02% ethanolic *p*-dimethylaminocinnamaldehyde containing 10% (v/v) of 2 *N* H_2SO_4.
D_5 = Reducing reagent (D_4 reagent followed by 1.5% (w/v) $Ti_2(SO_4)_3$ in *N* H_2SO_4).

Compound	R_F		Colour*				
	S_1	S_2	D_1	D_2	D_3	D_4	D_5
m-Dinitrobenzene	0.95	0.93	—	—	—	n	v
m-Nitrosonitrobenzene	0.96	0.93	—	—	—	pv	v
m-Nitrophenylhydroxylamine	0.97	0.92	—	—	—	pv	v
3,3′-Dinitroazoxybenzene	0.97	0.92	—	—	—	n	v
m-Nitroaniline	0.89	0.91	o	—	—	v	v
m-Phenylenediamine	0.05	0.20	—	—	—	v	v
2,4-Dinitrophenol	0.93	0.73	y	n	y	n	v
2,6-Dinitrophenol	0.91	0.71	y	n	y	n	v
3,5-Dinitrophenol	0.58	0.85	y	n	y	n	v
2-Amino-4-nitrophenol	0.08	0.75	o	bn	o	v	v
2-Amino-6-nitrophenol	0.86	0.83	o	bn	v	v	v
3-Amino-5-nitrophenol	0.07	0.74	y	bu	v	v	v
4-Amino-2-nitrophenol	0.56	0.82	r	bn	v	v	v
2,4-Diaminophenol	0.00	0.01	b	r	—	v	v
2,6-Diaminophenol	0.00	0.03	bn	bn	—	v	v
3,5-Diaminophenol	0.00	0.00	bn	bn	—	v	v

* o = orange; y = yellow; r = red; b = blue; bn = brown; bu = buff; n = none; v = violet; p = pale.

TABLE 38

R_F VALUES OF SOME COMPOUNDS RELATED TO PHENYLALANINE MUSTARD

(A. P. MARTINEZ, W. A. SKINNER, W. W. LEE, L. GOODMAN AND B. R. BAKER *J. Am. Chem. Soc.*, 82 (1960) 6050)

Solvent: S_1 = Benzene–methanol–water (2:6:1).
Paper: P_1 = Schleicher & Schüll No. 2495 acetylated paper.
P_2 = Whatman No. 1.
(Descending).
Detection: U.V. light.

Compound	R_F	
	S_1P_1	S_1P_2
Methyl *p*-amino-α-benzamidocinnamate	0.60	
p-Aminophenylpyruvic acid hydrochloride	0.72	0.67
Methyl α-benzamido-*p*-[bis-(2-hydroxyethyl)-amino]-cinnamate	0.79	
4-{*p*-[Bis-(2-chloroethyl)-amino]-benzylidene}-2-phenyl-2-oxazolin-5-one	0.24	
Methyl α-benzamido-*p*-[bis-(2-chloroethyl)-amino]-cinnamate	0.63	
p-[Bis-(2-chloroethyl)-amino]-phenylpyruvic acid	0.81*	

* Streaked in most runs.

TABLE 39

R_F VALUES OF SOME DERIVATIVES OF MELPHALAN [*p*-BIS-(2-CHLOROETHYL)-AMINO-L-PHENYLALANINE]

(F. BERGEL AND J. A. STOCK, *J. Chem. Soc.*, (1960) 3658)

Solvents: S_1 = *n*-Butanol–ethanol–propionic acid–water (10:5:2:5).
S_2 = 1% aqueous NH_4Cl.
S_3 = 2% aqueous NH_4Cl.
Paper: Whatman No. 1 (ascending).
Detection: 0.25% ninhydrin in acetone.

Compound	R_F*		
	S_1	S_2	S_3
Gly·Mel·OEt	0.80	—	0.52
L-Ala·Mel·OEt	0.89 (0.95)	0.69	—
L-Val·Mel·OEt	0.84	—	0.51
L-Phe·Mel·OEt	0.86	—	0.01
(L-Cys·Mel·OEt)$_2$ S	—	0.01	—
Mel·OEt	0.84 (0.94)	0.69	0.63
Gly·OEt	0.57	—	0.95
DL-Phe·OEt	0.87	—	—
DL-Ala·OEt	(0.66)	0.97	—
Mel	0.69	—	—
Val	0.38	—	—
Phe·Gly·OEt	0.81	—	—
Gly·Gly·OEt	0.57	—	—
Val·Mel	0.88	—	—
Gly·Phe·OEt	0.75	—	—

* R_F values for freshly prepared solvent given in parentheses.

TABLE 40

R_F VALUES OF SOME N,N-BIS-(2-CHLOROETHYL)-AMINOPHENYL-AMINO ACIDS

(T. A. CONNORS, W. C. J. ROSS AND J. G. WILSON, *J. Chem. Soc.*, (1960) 2994)

Solvents: S_1 = Butan-1-ol–ethanol–water–propionic acid (10:5:5:2).
S_2 = Water satd. butan-1-ol.
Paper: Whatman No. 1.
Detection: U.V. light (presumed).

Compound	R_F	
	S_1	S_2
α-[*m*-Bis-(2-chloroethyl)-aminophenyl]-alanine	0.80	—
1-Amino-7-[bis-(2′-chloroethyl)-amino]-1,2,3,4-tetrahydro-1-naphthoic acid	0.82	—
o-[Bis-(2-chloroethyl)-amino]-DL-phenylalanine	0.79	0.64
m-[Bis-(2-chloroethyl)-amino]-DL-phenylalanine	0.75	0.55
p-[Bis-(2-chloroethyl)-amino]-DL-phenylalanine	0.73	0.51

TABLE 41

R_F VALUES OF NAPHTHALENE AMINO-ACID CONJUGATES AND RELATED DECOMPOSITION PRODUCTS

(E. BOYLAND, G. S. RAMSAY AND P. SIMS, *Biochem. J.*, 78 (1961) 376)

Solvents: S_1 = Butan-1-ol–propan-1-ol–aq. 2 *N* NH_4OH (2:1:1, by vol.).
S_2 = Butan-1-ol–acetic acid–water (2:1:1, by vol.).
Paper: Whatman No. 1 (descending).
Time of run: 18 h.
Detection: D_1 = U.V. light fluorescence.
D_2 = Freshly diazotised *p*-nitroaniline (0.02% in 0.1 *N* HCl), followed by aq. 10% Na_2CO_3.
D_3 = 0.2% ninhydrin in acetone (70° for 10 min).
D_4 = 0.1 *M* $K_2Cr_2O_7$ in acetic acid (1:1, v/v), followed by 0.1 *M* $AgNO_3$ (R. H. KNIGHT AND L. YOUNG, *Biochem. J.*, 70 (1958) 111).

Compound	R_F		*Colour**			
	S_1	S_2	D_1	D_2	D_3	D_4
N-Acetyl-S-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0.37	0.84	d–a	b	—	+
1-Naphthylmercapturic acid	0.51	0.86	p**	—	—	+
S-(1,2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0.31	0.63	d–a	b	pu	+
S-(1-Naphthyl)-L-cysteine	0.46	0.75	p	—	pu	+
S-(1,2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine	0.25	0.62	d–a	b	bn–pu	+
S-(1-Naphthyl)-L-cysteinylglycine	0.40	0.74	p	—	bn–pu	+
S-(1,2-Dihydro-2-hydroxy-1-naphthyl)-glutathione	0.10	0.47	d–a	b	pu	+
S-(1-Naphthyl)-glutathione	0.21	0.67	p**	—	pu	+
Unknown I***	0.05	0.40	d–a	b	pu	+
Unknown II***	0.01	0.35	d–a	b	pu	+

---

* d = dark; a = absorption; b = blue; bn = brown; pu = purple; y = yellow; p = pink.
** After exposure to NH_3 fumes.
*** Perhaps taurine conjugates of S-(1,2-dihydro-2-hydroxy-1-naphthyl)-glutathione.

TABLE 42

R_F VALUES OF SOME S-CYSTEINE DERIVATIVES

(E. Boyland, G. S. Ramsay and P. Sims, *Biochem. J.*, 78 (1961) 376)

Solvents: S_1 = Butan-1-ol–propan-1-ol–aq. 2 *N* NH_4OH (2:1:1, by vol.).
S_2 = Butan-1-ol–acetic acid–water (2:1:1, by vol.).
Paper: Whatman No. 1 (descending).
Time of run: 18 h.
Detection: D_1 = 0.2 % ninhydrin in acetone (70°; 10 min).
D_2 = $K_2Cr_2O_7$–$AgNO_3$ reagent (0.1 *M* $K_2Cr_2O_7$–acetic acid (1:1, v/v) followed by 0.1 *M* $AgNO_3$; R. H. Knight and L. Young, *Biochem. J.*, 70 (1958) 111).

Compound	R_F		D_1	D_2
	S_1	S_2	*Colour**	*Result*
N-Acetyl-S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine**	0.56	0.88	—	+
Unknown***	0.39	0.79	—	+
S-(1,2,3,4-Tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine**	0.57	0.72	p	+
S-(1,2,3,4-Tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine**	0.37	0.72	b→p	+
S-(1,2,3,4-Tetrahydro-2-hydroxy-1-naphthyl)-glutathione**	0.25	0.45	p	+

* p = purple; b→p = brown turning purple.
** Probable identity.
*** Possible structures: N-acetyl-S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine, or (more probably) the N-acetylglycylcysteine derivative.

TABLE 43

R_F VALUES OF SOME NAPHTHOLS AND OTHER NAPHTHALENE METABOLITES

(E. Boyland, G. S. Ramsay and P. Sims, *Biochem. J.*, 78 (1961) 376)

Solvents: S_1 = Butan-1-ol–propan-1-ol–aq. 2 *N* NH_4OH (2:1:1, by vol.).
S_2 = 0.1 *N* NH_4OH.
Paper: Whatman No. 1 (descending, S_1; ascending, S_2).
Time of run: 18 h (S_1), 8 h (S_2).
Detection: D_1 = U.V. fluorescence.
D_2 = Freshly diazotised *p*-nitroaniline (0.02 % in 0.1 *N* HCl), followed by aq. 10 % Na_2CO_3.
D_3 = D_2 after spraying with 2 *N* HCl (100° for 10 min).

Compound	R_F		*Colour**		
	S_1	S_2	D_1	D_2	D_3
1-Naphthol	0.97	0.62	b	b	b
2-Naphthol	0.97	0.55	v	o	o
trans-1,2-Dihydro-1,2-dihydroxynaphthalene**	0.89	—	d–a	—	b
1-Naphthylglucosiduronic acid	0.28	—	d–v	—	b
trans-1,2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid**	0.18	—	d–a	p–o***	b
trans-1,2-Dihydro-1-hydroxy-2-naphthylglucosiduronic acid**	0.18	—	d–a	b***	b
2-Hydroxy-1-naphthylglucosiduronic acid	0.15	—	br–b	y	pu
1-Hydroxy-2-naphthylglucosiduronic acid	0.14	—	br–b	b	pu

* d = dark; a = absorption; b = blue; p = pale; br = bright; v = violet; o = orange; pu = purple; y = yellow.
** Optical isomers not separated.
*** Due to decomposition products.

TABLE 44

R_F VALUES OF SOME METABOLITES OF 1,2-DIHYDRONAPHTHALENE AND 1,2-EPOXY-1,2,3,4-TETRAHYDRONAPHTHALENE

(E. BOYLAND AND P. SIMS, *Biochem. J.*, 77 (1960) 175)

Solvents: S_1 = Butanol saturated with aq. 2 *N* NH_4OH.
S_2 = Butanol–propan-1-ol–aq. 2 *N* HN_4OH (2:1:1, by vol.).
S_3 = Butanol–acetic acid–water (12:3:5, by vol.).
S_4 = Butanol–acetic acid–water (2:1:1, by vol.).
Paper: Whatman No. 1 (descending).
Time of run: 18 h.
Detection: U.V. light; (A) freshly diazotised *p*-nitroaniline (0.02 % in 0.1 *N* HCl) followed by aq. 10 % Na_2CO_3; (B) 0.1 *M* $K_2Cr_2O_7$–acetic acid (1:1) followed by 0.1 *M* $AgNO_3$[a]; (C) aq. 2 % (w/v) $NaIO_4$ followed, after 30 min, by Schiff's reagent[b]; (D) platinic iodide reagent[c]; (E) ninhydrin in acetone (0.2 %) then heated to 70° for 10 min. (First three: spray; last two: dip.)

Compound	R_F				Colour[d]		
	S_1	S_2	S_3	S_4	*B*	*C*	*E*
S-(2-Hydroxy-1,2,3,4-tetrahydro-1-naphthyl)-L-cysteine	0.23	0.57	0.53	0.72	+	b[e]	p
N-Acetyl-S-(2-hydroxy-1,2,3,4-tetrahydro-1-naphthyl)-L-cysteine	0.25	0.56	0.81	0.88	+	b[e]	·
Methyl ester of N-acetyl-S-(2-hydroxy-1,2,3,4-tetrahydro-1-naphthyl)-L-cysteine	0.85	0.91	0.88	0.91	+	b[e]	·
trans-1,2-Dihydroxy-1,2,3,4-tetrahydronaphthalene	0.79	0.87	0.85	0.86	—	p→b	·
Glucosiduronate of *trans*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene	0.04	0.31	0.44	0.59	—	pk[f]	·

[a] R. H. KNIGHT AND L. YOUNG, *Biochem. J.*, 70 (1958) 111.
[b] C. J. W. BROOKS AND L. YOUNG, *Biochem. J.*, 63 (1956) 264.
[c] G. TOENNIES AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 823.
[d] p = purple; b = blue; p→b = purple turning blue; pk = pink; + = positive result; — = negative result; · = not tested (presumed).
[e] After 4 h. The reaction was not sensitive for small amounts of material.
[f] After 15 min.

TABLE 45

R_F VALUES OF FLAVONOID CONSTITUENTS OF *Melicope mantelli* BUCH. AND RELATED COMPOUNDS

(R. C. CAMBIE, *J. Chem. Soc.*, (1960) 2376)

Solvent: 1 % NH_4OH soln.–dioxan–light petroleum (1:1:1, upper phase).
Paper: Whatman No. 1.
Detection: NH_3 vapour; Dragendorff reagent, then with 5 % aq. $FeCl_3$.

Compound	R_F
Melisimplexin	0.73
Melisimplin	0.83
Meliternatin	0.42
Meliternin	0.17
Ternatin	0.63
Wharangin	0.09

TABLE 46

R_F VALUES OF VARIOUS FLAVAN DERIVATIVES

(J. W. CLARK-LEWIS, G. F. KATEKAR AND P. I. MORTIMER, *J. Chem. Soc.*, (1961) 499)

Solvents: S_1 = Forestal solvent: 2% acetic acid (E. C. BATE-SMITH, *Biochem. J.*, 58 (1954) 122).
S_2 = Butan-1-ol–acetic acid–water (4:1:5; S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238).
Paper: Not stated.
Detection: Not specified.

Compound	R_F	
	S_1	S_2
Teracacidin	0.46–0.54	
	0.41–0.52	
Isoteracacidin	0.58–0.68	
	0.58–0.64	
O-Ethylisoteracacidin	0.75–0.87	
	0.78–0.85	
	0.67–0.76	
Anthocyanidin	0.74	
Cyanidin	0.55	
3,7,8,3′,4′-Pentahydroxyflavylium chloride	0.58	
Pinitol		0.23
Inositol		0.14

TABLE 47

R_F VALUES OF MELACACIDIN, ISOMELACACIDIN AND RELATED FLAVAN DERIVATIVES

(J. W. CLARK-LEWIS AND P. I. MORTIMER, *J. Chem. Soc.*, (1960) 4106)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5) (S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238).
S_2 = 2% acetic acid (aqueous).
S_3 = Water–acetic acid–conc. HCl (10:30:3) (E. C. BATE-SMITH, *Biochem. J.*, 58 (1954) 122).
Paper: Not given.
Detection: D_1 = U.V. light except where otherwise indicated.
D_2 = HCl.
D_3 = Alcoholic 3% toluene-*p*-sulphonic acid.
D_4 = Alcoholic $AlCl_3$.

Compound	R_F			Colour*			
	S_1	S_2	S_3	D_1	D_2	D_3	D_4
Dihydro-(?,7,8,3′,4′)-tetrahydroxyflavonol	0.57–0.79			fb fl; y**	dy	dy	
Okanin	0.52***						
Melacacidin	0.25–0.32,	0.30–0.42,	0.58				
	0.42–0.48	0.35–0.47					
O-Methylisomelacacidin	0.63–0.73	0.53–0.66					
O-Ethylisomelacacidin	0.73–0.82	0.63–0.74					
		0.58–0.70					+
Cyanidin			0.55				

* f = faint; b = blue; fl = fluorescence; d = deep; y = yellow.
** Visible light; after 3–4 h.
*** R_F' (*i.e.* leading edge).

TABLE 48

R_F VALUES OF MATTEUCININ AND OTHER ERICACEAE CONSTITUENTS

(H. R. ARTHUR AND S. W. TAM, *J. Chem. Soc.*, (1960) 3197)

Solvent: Phenol saturated with water.
Paper: Whatman No. 1.
Temperature of run: T_1 = ~ 20°; T_2 = ~ 30°; T_3 = ~ 25°; T_4 = 22°; T_5 = 15°.
Detection: $FeCl_3$ soln.; ammonium molybdate (for glucose).

Compound	R_F				
	T_1	T_2	T_3	T_4	T_5
Quercetin	0.42	0.54			
Aromadendrin			0.83		
Myricetin	0.21	0.34			
Farrerol	0.93				
Matteucinol	0.95				
Matteucinin	0.89				
D-Glucose				0.46	0.36

TABLE 49

R_F VALUES OF FLAVANONES FROM *Angophora lanceolata* AND RELATED DEGRADATION PRODUCTS

(A. J. BIRCH, D. G. PETIT, A. J. RYAN AND R. N. SPEAKE, *J. Chem. Soc.*, (1960) 2063)

Solvents: S_1 = Butanol–acetic acid–water (G. LINDSTED AND A. MISIORNY, *Acta Chem. Scand.*, 5 (1951) 1).
S_2 = Butanol–water (G. LINDSTET AND A. MISIORNY, *Acta Chem. Scand.*, 5 (1951) 1).
S_3 = Benzene–ligroin–methanol–water (G. LINDSTEDT AND A. MISIORNY, *Acta Chem. Scand.*, 5 (1951) 1).
Paper: Whatman No. 1.
Detection: D_1 = U.V. light after NH_3 fumes.
D_2 = Bisdiazotised benzidine reagent.
D_3 = Diazotised *p*-nitroaniline.

Compound	R_F			Colour*		
	S_1**	S_2	S_3	D_1	D_2	D_3
2,4-Dimethylphloroglucinol	0.94			b		
Phloroglucinol	0.75					
2-Methylphloroglucinol	0.79					
p-Hydroxybenzoic acid		0.15				r
4-Hydroxycinnamic acid		0.22				i-b
Methylmatteucinol			0.95		bk-r	
Farrerol			0.3-0.4		p-c	
Matteucinol			0.85		c	
Angophorol			0.85		w	
Unknown***			0.75		pi	

* b = blue; r = red; i = indigo; bk = brick; p = pale; c = cream; w = yellow; pi = pink.
** Presumed from text.
*** Possibly isomer of angophorol.

TABLE 50

R_F VALUES OF THE ALKALOID DAPHNANDRINE AND RELATED COMPOUNDS

(I. R. C. BICK, P. S. CLEZY AND M. J. VERNENGO, *J. Chem. Soc.*, (1960) 4928)

Solvents: S_1 = Butan-1-ol–acetic acid–water (63:10:27).
S_2 = Butan-1-ol–acetic acid–water (4:1:5).
Paper: Whatman No. 1.
Detection: Not given.

No.	*Compound*	R_F S_1	R_F S_2
1	Daphnandrine	0.48	
2	O-Ethyldaphnandrine	0.56	
3	O-Ethylcoclaurine*		0.79
4	O-Ethyl-N-methylcoclaurine*		0.88
5	O-Ethylcoclaurine methiodide**		0.84
6	O-Ethyl-N-methylcoclaurine methiodide**		0.84

* Order of No. 3 and 4, and hence 5 and 6, presumed (not specified).
** No resolution.

TABLE 51

R_F VALUES OF KREYSIGINE, FLORAMULTINE, KREYSIGININE AND FLORAMULTININE (ALKALOIDS FROM *Kreysigia multiflora* REICHB).

(G. M. BADGER AND R. B. BRADBURY, *J. Chem. Soc.*, (1960) 445)

Solvent: 5% Aqueous acetic acid–butan-1-ol (1:1, v/v; upper phase).
Paper: Not specified.
Temperature of run: 15°.
Detection: With iodine.

Compound	R_F	*Colour**
Kreysigine	0.55	py
Floramultine	0.51	py
Kreysiginine	0.40	py → do
Floramultinine	0.30	py → do

* py = pale yellow; do = deep orange; → = after several hours in air.

TABLE 52

R_F VALUES OF SOME VITAMINS AND RELATED COMPOUNDS

(E. E. Gadsen, C. H. Edwards and G. A. Edwards, *Anal. Chem.*, 32 (1960) 1415)

Solvents: S_1 = Phenol–citrate/phosphate buffer (100:25, v/v; aqueous buffer: 6.3% sodium citrate, 3.7% KH_2PO_4).

S_2 = Butan-1-ol–propionic acid–water (freshly prepared from equal vols. of solution A (1246 ml butan-1-ol + 84 ml water) and solution B (620 ml propionic acid + 790 ml water)).

Paper: Whatman No. 1 (descending).

Time of run: 18–22 h (S_1); 14–16 h (S_2).

Temperature of run: 24° ± 0.5°.

Detection: D_1 = Ammoniacal silver nitrate[a].
D_2 = Ferricyanide–nitroprusside[b].
D_3 = Ninhydrin.
D_4 = Iodine vapour.
D_5 = 2,6-Dichlorophenolindophenol[b].
D_6 = Cyanogen bromide[a].
D_7 = Ferric chloride[b].
D_8 = Phenol–hypochlorite reagent[b].
D_9 = Light.

Order for multiple detection: D_9 (UV), D_8, D_4, D_7 or D_1.

Compound	R_F		Quantity used	Colour[c]								
	S_1	S_2	µg	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
Vitamin A	0.90	—	250									y[e]
α-Tocopherol	0.89	0.77	250[d]	b								
Menadione	0.94	—	250									y[e]
Thiamine	0.93	0.55	250		f	y						
Riboflavine	0.91	0.32	20									y, f
Niacin	0.83	0.68	250				br	p	o			
Nicotinamide	0.85	0.69	250		y		br		y			
Pyridoxine	0.87	0.60	250							br		f
Pantothenic acid	0.66	0.38	250			pu						
Biotin	0.79	0.78	250				br					
Inositol	0.21	0.12	50	br								
Choline	0.87	0.52	250				br					
p-Aminobenzoic acid	0.80	0.69	250							br	br	
Folic acid	0.34	0.30	125									f
Vitamin B_{12}	0.92	0.31	50									p
Vitamin C	0.34	0.34	250	br								

[a] R. J. Block, E. L. Durrum and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press Inc., New York, 1958, pp. 398–400.

[b] Biochemical Institutes Studies IV, *Univ. Texas Publ.*, No. 5109 (1951).

[c] y = yellow; b = black; br = brown; p = pink; o = orange; pu = purple; f = fluorescent in U.V.

[d] In mg.

[e] After phenol run only.

TABLE 53

R_F VALUES (RELATIVE) OF SOME HEXITOLS AND THEIR ACETALS

(T. G. BONNER AND N. M. SAVILLE, *J. Chem. Soc.*, (1960) 2851)

Solvents: S_1 = Butan-1-ol satd. with H_2O (at 0°).
S_2 = Acetone–water (4:1; v/v).
S_3 = Butan-1-ol–pyridine–water–satd. aq. boric acid (6:4:2:1; by vol.).

Paper: Whatman No. 1.

Detection: Reagents as in literature (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444; M. L. WOLFROM AND J. B. MILLER, *Anal. Chem.*, 28 (1956) 1037; L. HOUGH, J. K. N. JONES AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702; K. WALLENFELS, *Naturwiss.*, 37 (1950) 491).

Compound	R_G*	R_M**		
	S_2	S_1	S_2	S_3
D-Glucitol	1.00			
2,4-O-Methylene-D-glucitol	1.25, 1.36			
D-Mannitol		1.00	1.00	1.00
2,5-O-Methylene-D-mannitol			1.28	2.00
1,3-O-Methylene-D-mannitol		2.66	1.40	
2-O-Methyl-D-mannitol		2.60		1.41

* R_G = R_F of compound/R_F of glucitol.
** R_M = R_F of compound/R_F of mannitol.

TABLE 54

R_F VALUES OF SOME PENTAERYTHRITOL ESTERS AND RELATED COMPOUNDS

(T. G. BONNER, E. J. BOURNE AND N. M. SAVILLE, *J. Chem. Soc.*, (1960) 2917)

Solvents: S_1 = Water satd. butan-1-ol (at 0°).
S_2 = Light petroleum (b.p. 60–80°) satd. with dimethyl sulphoxide (B. WICKBERG, *Acta Chem. Scand.*, 12 (1958) 615).

Paper: Whatman No. 1.

Impregnation: I_1 = Dip paper in 20% dimethyl sulphoxide in benzene, press between absorbent paper, dry 1 min at 60°. Repeat treatment and place between glass plates to prevent water absorption. Dry at 120° for 20 min before spraying.

Detection: D_1 = Hydroxylamine reagent (M. ABDEL-AKHER AND F. SMITH, *J. Am. Chem. Soc.*, 73 (1951) 5859) [freshly prepared 1:1 v/v mixture of methanolic hydroxylamine hydrochloride (6.95 g/100 ml) and KOH (6.17 g/100 ml)].
Time and temperature of drying depending on ester reactivity, *e.g.* pentaerythritol tetraacetate is detected only by heating to 110° for 10 min.
Final spraying: 1:1 v/v mixture of aqueous $FeCl_3$ and aqueous 0.5 N HCl. (Acetates and lactones give purple spots on yellow background, benzoates not detected).

D_2 = 2,4-Dinitrophenylhydrazine spray (S. A. BARKER, E. J. BOURNE, A. B. FOSTER AND R. M. PINKHARD, *Chem. & Ind.* (*London*), (1959) 226). (Detection of benzylidene and isopropylidene derivatives.)

Compound	R_F	
	S_1	S_2I_1
Tetra-O-acetylpentaerythritol	0.94	0.49, 0.51
Pentaerythritol	0.45	
Di-O-acetyl-O-benzylidenepentaerythritol		0.66, 0.68, 0.71
Mono-O-acetyl-O-benzylidenepentaerythritol*		0.05

* Possible identification.

TABLE 55

R_F VALUES OF SOME POLYHYDRIC ALCOHOLS AND RELATED COMPOUNDS

(A. B. Foster, D. Horton, N. Salim, M. Stacey and J. M. Webber, *J. Chem. Soc.*, (1960) 2587)

Solvents: Butanol–ethanol–water (4:1:5), organic phase.
Paper: Whatman No. 1 (descending).
Detection: Aniline hydrogen phthalate, ninhydrin and $AgNO_3$ reagents.

Compound	R_F
2-Acetamido-2-deoxy-3-O-methyl-D-glucitol	0.31
Glycerol	0.43
Erythritol	0.315
D-Arabitol	0.225
D-Glucitol	0.145
2-Amino-2-deoxyglycerol HCl	0.17
2-Amino-2-deoxy-D-threitol HCl	0.14
2-Amino-2-deoxy-D-xylitol HCl	0.12
2-Amino-2-deoxy-D-glucitol HCl	0.097

TABLE 56

R_G VALUES (RELATIVE) OF SOME ACYCLIC POLYHYDROXY COMPOUNDS AND THEIR PRE-FORMED MOLYBDATE COMPLEXES

(E. J. Bourne, D. H. Hutson and H. Weigel, *J. Chem. Soc.*, (1961) 35)

Solvent: Butan-1-ol–acetic acid–water (4:1:5), organic phase.
Paper: Whatman No. 1.
Detection: D_1 = Acetone–silver nitrate–alcoholic sodium hydroxide (W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, 166 (1950) 444); for polyhydroxy compounds.
D_2 = 5% aqueous catechol solution (J. B. Pridham, *J. Chromatog.*, 2 (1959) 605); for molybdate-containing compounds.

Preparation of pre-formed complexes: aqueous polyhydroxy compound (1 mol.) mixed with aqueous sodium molybdate (2 mol.), pH adjusted to 2 with IR-120 (H^+).

Original compound	R_G Untreated compound	R_G Pre-formed complexes			
Sorbitol	1.0	0.9*	0.6*†	0.5*†	0†
2-Deoxysorbitol	1.2	1.2*	0.7*†	0.4*†	0†
D-Mannitol	1.1	0.9*	0.7*†	0.5*†	0†
Galactitol	1.1	1.1*	0.7*†	0.5*†	0†
D-Arabitol	1.1	1.1*	0.7*†	0.4*†	0†
Erythritol	1.7	1.7*	1.2*†	—	0†
Glycerol	2.2	2.2*	—	—	0†
Molybdic acid	—	—	—	—	0†

R_G = R_F compound/R_F glucose.
* D_1.
† D_2.

TABLE 37

ELECTROPHORETIC MOBILITIES OF ACYCLIC POLYHYDROXY COMPOUNDS

(E. J. BOURNE, D. H. HUTSON AND H. WEIGEL, *J. Chem. Soc.*, (1961) 35)

Electrolyte: Hydrated sodium molybdate in water (25 g in 1200 ml) adjusted to pH 5 with H_2SO_4.
Paper: Whatman No. 3 MM (10 cm wide).
Apparatus: High voltage; horizontal (D. GROSS, *Chem. & Ind. (London)*, (1959) 1219).
Potential: 20–80 V/cm (not more closely defined).
Time of run: 1–2 h (not more closely defined).
Units: $M_S = \dfrac{\text{True distance migrated by compound}}{\text{True distance migrated by sorbitol}}$.
Detection: Acetone–silver nitrate–alcoholic sodium hydroxide (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444); 0.1 N H_2SO_4 spray followed by heating (120°; 10 min) for primary hydroxyl-containing compounds (bluish green spots).

Compound	M_S	*Compound*	M_S
Ethane-1,2-diol	<0.1	2,3-Di-O-methylsorbitol	<0.1
Propane-1,2-diol	<0.1	4-O-α-D-Glucopyranosylsorbitol	0.4
Propane-1,3-diol	<0.1	4-O-β-D-Glucopyranosylsorbitol	0.4
Butane-2,3-diol	<0.1	4-O-β-D-Galactopyranosylsorbitol	0.4
Butane-1,3-diol	<0.1	4-O-α-Isomaltosylsorbitol	0.4
Butane-1,4-diol	<0.1	4-O-α-Nigerosylsorbitol	0.4
Pentane-1,5-diol	<0.1	5-O-α-D-Glucopyranosylsorbitol	0.8
Hexane-1,6-diol	<0.1	6-O-α-D-Glucopyranosylsorbitol	0.8
2-Methylpentane-2,4-diol	<0.1	6-O-β-D-Glucopyranosylsorbitol	0.8
2-Methylhexane-1,3-diol	<0.1	6-O-α-D-Galactopyranosylsorbitol	0.8
Pentaerythritol	<0.1	6-O-α-Isomaltosylsorbitol	0.7
Glycerol	<0.1	6-O-α-Isomaltotriosylsorbitol	0.6
Erythritol	1.0	6-O-α-Isomaltotetraosylsorbitol	0.5
D-Threitol	0.5	6-O-α-Isomaltopentaosylsorbitol	0.4
Ribitol	1.1	6-O-α-Isomaltohexaosylsorbitol	0.3
D-Arabitol	1.1	6-O-α-Isomaltoheptaosylsorbitol	0.25
3-O-α-D-Galactopyranosyl-D-arabitol	<0.1	D-Mannitol	1.0
		1-Deoxy-D-mannitol	1.0
Xylitol	1.1	2-O-Methyl-D-mannitol	1.0
Sorbitol	1.0	2-O-α-D-Glucopyranosyl-D-mannitol	0.8
2-Deoxysorbitol	1.0	2-O-α-D-Mannopyranosyl-D-mannitol	0.8
2-O-β-D-Glucopyranosylsorbitol	0.9	3-O-α-Mannopyranosyl-D-mannitol	<0.1
2-O-Methylsorbitol	<0.1	1,2-Di-O-methyl-D-mannitol	1.0
3-O-α-D-Glucopyranosylsorbitol	<0.1	Galactitol	1.0
3-O-β-D-Glucopyranosylsorbitol	<0.1	6-Deoxy-D-galactitol	1.0
3-O-α-Maltosylsorbitol	<0.1		

TABLE 58

ELECTROPHORETIC MOBILITIES OF POLYHYDROXY COMPOUNDS

(E. J. Bourne, D. H. Hutson and H. Weigel, *J. Chem. Soc.*, (1960) 4252)

Electrolyte: Aq. sodium molybdate dihydrate (25 g/1200 ml), adjusted to pH 5 with conc. H_2SO_4.
Paper: Whatman No. 3 MM (10 cm wide).
Apparatus: Gross type (max. 3000 V at 100 mA); horizontal, cooled.
Potential: 30–60 V/cm.
Time: 1–2 h.
Units: M_S = Migration relative to sorbitol (*ca.* 25 cm in 2 h at 60 V/cm).
Marker: Glycerol (comparison with 2,3,4,6-tetra-O-methyl-D-glucose showed that it did not form a complex; used to correct for electroendosmosis).
Detection: Acetone-silver nitrate-ethanolic sodium hydroxide (W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, 166 (1950) 444); *p*-anisidine hydrochloride in butan-1-ol (L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, (1950) 1702).

Compound	M_S	*Compound*	M_S
Complex forming			
Aldoses and their derivatives		*Cyclitols*	
D-Erythrose	0.9	Myoinositol	0.2
L-Threose	0.6	Alloinositol	0.4
D-Ribose	0.4	Epi-inositol	1.1
Methyl α-D-ribopyranoside	0.1		
D-Lyxose	1.1	*1,6-Anhydro-β-D-aldopyranoses*	
D-Mannose	0 → 0.9	1,6-Anhydro-β-D-mannopyranose	0.5
D-Gulose	1.1		
D-Talose	0.7		
L-Rhamnose	0 → 0.6	*Ketoses*	
D-Glycero-D-alloheptose	0.9	D-Fructose	0.5
D-Glycero-L-glucoheptose	0.2	L-Sorbose	0.3
D-Glycero-L-mannoheptose	0.8	D-Glucosone	0.9
D-Glycero-D-guloheptose	1.1	D-Glucoheptulose	1.0
D-Glycero-D-idoheptose	1.0	D-Mannoheptulose	0.4
D-Glycero-D-galaheptose	0.4	Leucrose	0.4
D-Glycero-L-galaheptose	0.4	Turanose	0.1
Not complex forming			
Aldoses and their derivatives		*Aldoses and their derivatives*	
Glyceraldehyde	<0.1	Methyl α-D-glucopyranoside	<0.1
2-Deoxy-D-ribose	<0.1	Phenyl β-D-glucopyranoside	<0.1
D-Arabinose	<0.1	Catechol β-D-glucopyranoside	<0.1
Methyl α-D-arabopyranoside	<0.1	3,4-Di-O-methyl-D-mannose	<0.1
Methyl β-D-arabopyranoside	<0.1	Methyl α-D-mannopyranoside	<0.1
1,2-Dideoxy-D-arabinose	<0.1	Methyl β-D-mannopyranoside	<0.1
D-Xylose	<0.1	D-Galactose	<0.1
Methyl α-D-xylofuranoside	<0.1	Sophorose	<0.1
Methyl α-D-lyxopyranoside	<0.1	Nigerose	<0.1
Methyl β-D-lyxopyranoside	<0.1	Laminaribiose	<0.1
D-Altrose	<0.1	Maltose	<0.1
D-Glucose	<0.1	Cellobiose	<0.1
3-O-Methyl-D-glucose	<0.1	Lactose	<0.1
2,3,4-Tri-O-methyl-D-glucose	<0.1	Isomaltose	<0.1
2,3,6-Tri-O-methyl-D-glucose	<0.1	Gentiobiose	<0.1
2,3,4,6-Tetra-O-methyl-D-glucose	<0.1	Melibiose	<0.1
2-Deoxy-D-glucose	<0.1		

(*Continued on p. D37*)

TABLE 58 *(continued)*

Compound	M_S	*Compound*	M_S
		Not complex forming	
Cyclitols		*1,6-Anhydro-β-D-aldopyranoses*	
Mucoinositol	<0.1	1,6-Anhydro-β-D-glucopyranose	<0.1
Scylloinositol	<0.1	1,6-Anhydro-β-D-gulopyranose	<0.1
Mytilitol	<0.1	1,6-Anhydro-β-D-galactopyranose	<0.1
Pinitol	<0.1		
Quebrachitol	<0.1	*Ketose*	
(−)-Viboquercitol	<0.1	Sucrose	<0.1
Scylloquercitol	<0.1		
(−)-Protoquercitol	<0.1		

TABLE 59

ELECTROPHORETIC MOBILITIES OF SOME MONOMETHYLGLUCOSES

(T. G. Bonner, E. J. Bourne and S. McNally, *J. Chem. Soc.*, (1960) 2929)

Electrolyte: 0.2 *M* borate buffer (pH 10.0).
Paper: Whatman No. 3.
Apparatus: Not given*.
Potential: Not given*.
Units: M_G*.
Detection: AgNO$_3$ and ethanolic NaOH; *p*-anisidine–HCl; aniline hydrogen phthalate; urea hydrochloride; diphenylamine, aniline and phosphoric acid; 2,4-dinitrophenylhydrazine and HCl; α-naphthol and phosphoric acid; phloroglucinol and trichloroacetic acid; potassium periodatocuprate (T. G. Bonner, *Chem. & Ind. (London)*, (1960) 345).

Compound	M_G*
2-O-Methylglucose	0.23
3-O-Methylglucose	0.82
4-O-Methylglucose	0.24
6-O-Methylglucose	0.82

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* *Cf.* A. B. Foster, *J. Chem. Soc.*, (1953) 982.

TABLE 60

R_F VALUES OF SOME METHYLATED SUGARS

(P. D. Bragg and L. Hough, *Biochem. J.*, 78 (1961) 11)

Solvent: Butan-1-ol–ethanol–water (40:11:19, by vol.).
Paper: Whatman No. 1 (descending).
Detection: *p*-Anisidine hydrochloride (L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, (1950) 1702).

Compound	R_F
2,6-Di-O-methyl-D-mannose*	0.54–0.57
Mono-O-methylmannose**	0.36
2,3,4,6-Tetra-O-methyl-D-galactose	0.70–0.74
Tri-O-methylhexose**	0.61–0.63
2,3,4,6-Tetra-O-methyl-D-mannose	0.81–0.82
Tri-O-methylmannose**	0.71

* Tentative identification.
** Not identified closer.

TABLE 61

R_F VALUES (RELATIVE) OF SUGARS AND METHYLATED SUGARS

(G. O. Aspinall, M. J. Johnston and A. M. Stephen, *J. Chem. Soc.*, (1960) 4918)

Solvents: S_1 = Butan-1-ol–ethanol–water (4:1:5, upper layer).
S_2 = Benzene–ethanol–water (169:47:15, upper layer).
S_3 = Ethyl acetate–acetic acid–formic acid–water (18:3:1:4).
Paper: Whatman Nos. 1, 4 and 20 (not specified).
Detection: Spray of *p*-anisidine hydrochloride in moist butan-1-ol (L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, (1950) 1702).

Compound	R_G*		
	S_1	S_2	S_3
Mono-O-methylrhamnose	0.55, 0.58, 0.57		
2,3,4,6-Tetra-O-methyl-D-galactose	0.86, 0.89	0.87	
3,4-Di-O-methyl-L-rhamnose	0.86	0.25	
2,3,6-Tri-O-methyl-D-galactose	0.69, 0.70, 0.72	0.71	0.25
2,3,6-Tri-O-methyl-D-galactonolactone	1.00		
2,3,4-Tri-O-methyl-D-galactose	0.68, 0.65		
2,3,4-Tri-O-methyl-L-lyxose**	0.82		
2,3,4-Tri-O-methyl-L-rhamnose	1.00		
2,3,5-Tri-O-methyl-L-arabinose	0.98	1.02	
2,3-Di-O-methyl-L-threose**	0.92	0.90	
3-O-Methyl-L-rhamnose	0.58, 0.57, 0.55		
2,6-Di-O-methyl-D-galactose	0.49, 0.50, 0.48		
2,3-Di-O-methyl-D-galactose	0.45, 0.46, 0.48		
2,4-Di-O-methyl-D-galactose	0.43, 0.49, 0.44		
2,3,4-Tri-O-methyl-D-glucose	0.87, 0.88, 0.81	0.20	
2,3,4-Tri-O-methyl-L-xylose***	0.92	0.86	
3,4-Di-O-methyl-D-mannose		0.07	
Rhamnose	0.30		
2,3,4,6-Tetra-O-methyl-D-glucose	1.00		
2-O-Methyl-D-galactose	0.29		

* R_G = R_F compound/R_F of 2,3,4,6-tetra-O-methyl-D-glucose.
** Tentative identification.
*** Presumed.

TABLE 62

R_F VALUES (RELATIVE) OF SOME OLIGOSACCHARIDES AND METHYL SUGARS

(G. O. Aspinall, I. M. Cairncross, R. J. Sturgeon and K. C. B. Wilkie, *J. Chem. Soc.*, (1960) 3881)

Solvents: S_1 = Ethyl acetate–pyridine–water (10:4:3).
S_2 = Butan-1-ol–ethanol–water (4:1:5) upper layer.
Paper: Whatman No. 1 or 3 MM (not specified).
Detection: Not given.

Compound	R_{Xylose}* S_1	R_G** S_2
Xylobiose	0.60	
Xylotriose	0.39	
Xylotetraose	0.24	
O-L-Arabinofuranosyl(1→3)-O-β-D-xylopyranosyl(1→4)-D-xylose	0.40	
2,3,5-Tri-O-methyl-L-arabinose		0.96
2,4-Di-O-methyl-D-xylose		0.70

* R_{Xylose} = R_F compound/R_F xylose.
** R_G: Not defined.

TABLE 63

R_F VALUES OF SOME METHYL GLYCOSIDE PHOSPHATES

(P. Szabó and L. Szabó, *J. Chem. Soc.*, (1960) 3762)

Solvents: S_1 = Propan-2-ol–ammonia–water (7:1:2).
S_2 = Propan-1-ol–ammonia–water (7:1:2).
S_3 = Butan-1-ol–acetic acid–water (4:1:5).
S_4 = Propan-2-ol–conc. HCl–water (65:17.2:17.8).
S_5 = Propan-1-ol–ammonia–water (6:1:3).
Paper: Whatman No. 1 (ascending).
Detection: Not specified.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Me-α-D-glucoside 2-(dihydrogen phosphate)	0.29	0.18	0.15	—	0.48
Me-α-D-glucoside 3-(dihydrogen phosphate)	0.34	0.20	0.10	—	0.50
Me-α-D-glucoside 4-(dihydrogen phosphate)	0.32	0.18	0.15	—	0.45
Me-α-D-glucoside 6-(dihydrogen phosphate)	0.26	0.13	—	—	0.41
Me-β-D-galactoside 4-(dihydrogen phosphate)	—	0.21	0.17	—	0.45
Me-β-D-galactoside 6-(dihydrogen phosphate)	—	0.11	0.09	—	0.36
Me-α-D-glucoside 4,6-(hydrogen phosphate)	0.64	0.47	0.16	0.90	—
Me-α-D-glucoside 4,6-(hydrogen phosphate)	—	0.47	0.16	—	—
Me-β-D-galactoside 4,6-(hydrogen phosphate)	0.57	0.38	0.12	0.86	—

TABLE 64

R_F VALUES OF 1,2-O-ISOPROPYLIDENE-D-GLUCOFURANOSE PHOSPHATES

(P. Szabó and L. Szabó, *J. Chem. Soc.*, (1960) [illegible])

Solvents: S_1 = Propan-2-ol-ammonia-water (7:1:2).
S_2 = Propan-2-ol-ammonia-water (8:1:1).
Paper: Whatman No. 1 (ascending).
Detection: Not given.

Phosphate of 1,2-O-isopropylidene-D-glucofuranose	R_F	
	S_1	S_2
3-Phosphate	0.42	0.30
3,5-Phosphate	0.77	0.57; 0.66
3,6-Phosphate	0.72	0.66; 0.55
5-Phosphate	0.40	0.20
6-Phosphate	0.37	0.11

TABLE 65

R_F VALUES (RELATIVE) OF TRIPHENYLMETHYL ETHERS OF CERTAIN CARBOHYDRATES

(D. A. Applegarth and J. G. Buchanan, *J. Chem. Soc.*, (1960) [illegible])

Solvent: Di-isopropyl ether.
Paper: Whatman No. 1 (descending).
Impregnation: Paper dipped twice in 20% v/v solution of dimethyl sulphoxide in benzene and drying at 60° for 90 sec each time. Dimethyl sulphoxide removed after run by heating at 75° for 25 min (B. Wickberg, *Acta Chem. Scand.*, 12 (1958) 615).
Detection: D_1 = Spray of approx. N perchloric acid; heat at 75° for 5 min ([illegible] triphenylmethanol). Colour (yellow) fades on cooling, restored on heating (for triphenylmethyl group).
D_2 = Removal of triphenylmethyl group. Dip paper in ethanolic formic acid solution (25% v/v of 98% formic acid). Heat at 100° for 10 min. Place in forced draught at room temp. for 1 h. Alkaline silver nitrate (W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, 166 (1950) 444) or periodate-Schiff's reagent (J. Baddiley, J. G. Buchanan and B. Carss, *J. Chem. Soc.*, (1957) 4138) for polyols and similar compounds.

Compound	R_T*
2,3,4-Tri-O-acetyl-1,5-di-O-triphenylmethylribitol	1.27
Methyl 3,4-anhydro-6-O-triphenylmethyl-α-D-galactoside	0.63
Methyl 2,3-anhydro-6-O-triphenylmethyl-α-D-guloside	0.43
1,5-Di-O-triphenylmethylribitol	0.25
1-O-Triphenylmethylribitol	0.09

* R_T = R_F compound/R_F triphenylmethanol.
R_F of triphenylmethanol varied from 0.50 to 0.75 but rates of movement relative to triphenylmethanol were fairly constant.

TABLE 66

R_F VALUES OF PIPECOLIC ACIDS AND RELATED COMPOUNDS

J. W. CLARK-LEWIS AND P. I. MORTIMER, *J. Chem. Soc.*, (1960) 189.

Solvents: S_1 = Butanol–acetic acid–water (4:1:5; S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238).
S_2 = Butanol–benzyl alcohol (1:1).
S_3 = Phenol–water; ammonia and HCN atmosphere (R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224).

Paper: Not specified.

Time of run: T_1 = 12 h; T_2 = 16 h; T_3 = 15 h; T_4 = 40 h; T_5 = 24 h; T_6 = 19 h; T_7 = 19 h; T_8 = 44 h; T_9 = 20 h; T_{10} = 28 h; T_{11} = 48 h.

Length of run: L_1 = 24 cm; L_2 = 37 cm; L_3 = 30 cm; L_4 = 27.4 cm; L_5 = 33 cm; L_6 = 48 cm.

Impregnation: I_1 = M/15 phosphate buffer pH 7.5 (E. F. McFARREN, *Anal. Chem.*, 23 (1951) 168; L. F. BURROUGHS, *J. Sci. Food Agric.*, 8 (1957) 122).

Detection: D_1 = Ninhydrin at 100°.
D_2 = Isatin at 100°.
D_3 = Cold neutral silver nitrate solution.
D_4 = U.V. light, fluorescence.

Compound	R_F		R_P*		Colour**			
	S_1	S_2	S_1	S_2I_1	D_1	D_2	D_3	D_4
Pipecolic acid	$0.30^{a,i}$; 0.37^{b} 0.33^{c}; 0.33^{d}	0.33^{b}; 0.27^{e}	1.00^{g}	1.00^{f}	bl-v (S_2)	gn (S_2)		br
cis-4-Hydroxy-pipecolic acid *trans*-4-Hydroxy-pipecolic acid	0.16^{a}; $0.29^{a,i}$	0.53^{b}; 0.47^{e}	0.56^{g} 0.47^{g}	0.28^{f}	gn bl-p gn, gn-bn (S_2)	gn		du
4-Iodopipecolic acids	0.52^{a}; 0.58^{a}	—	—	—	p	bl-gn, fa-pi	bn	
2-Aminopent-4-enoic acid	0.37^{c}	—	0.90^{h}	0.50^{f}	bn (S_2) p^{1} v-gn^{2}	w (S_2) p-pi		
Baikiain	0.28^{a}; 0.31^{c}	—	0.86^{h}	0.82^{f}	br (S_2)	gn bk-r (S_2)	pi	
Proline	—	—	—	0.46^{f}	o (S_2)	bl (S_2)		
5-Hydroxypipecolic acid	—	0.53^{b}; 0.45^{e}	0.58^{k} 0.66^{m}	0.27^{f}	v-gr	gr		
2-Aminopentanoic acid (norvaline)	0.45^{j}; 0.49^{e}	—	—	—	p	pi		nil
Valine	0.39^{d}							
α-Aminobutyric acid	0.28^{d}	—	—	—				
3-Hydroxypyrrolidin-2-ylacetic acid	0.27^{m}				v-o	w		na
Leucine	0.54^{d}	—	—	—				
Isoleucine	0.54^{d}	—	—	—				
β-Alanine	0.24^{d}	—	—	—				
cis-3-Hydroxypipecolic acid	—	0.23^{e}	0.42^{k} 0.52^{m}					

---

* R_P = Distance moved by compound/distance moved by pipecolic acid.

** bk = brick red; bl = blue; bn = brown; br = bright; du = dull; eg = edge grey; fa = faint; gn = green; gr = grey; na = no absorption; o = orange; p = purple; pi = pink; r = red; v = violet; w = white; y = yellow.

a = L_2T_2; b = L_1T_1; c = L_3T_3; d = L_4T_4; e = L_5T_5; f = T_7; g = T_8; h = T_6; i = T_9; j = L_2T_{10}; k = T_{10}; m = T_{11}; n = L_6T_6; 1 = 110–115°; 2 = 80–90°.

TABLE 67

R_F VALUES OF AMINO ACIDS AND RELATED COMPOUNDS FROM OSTREOGRYCIN

(F. W. EASTWOOD, B. K. SNELL AND A. TODD, *J. Chem. Soc.*, (1960) 2286)

Solvents: S_1 = Phenol–water (4:1) in presence of HCN.
S_2 = 2,6-Lutidine–collidine–water (1:1:1) containing 1% diethylamine.
S_3, S_4, S_5 = described by T. L. HARDY, D. O. HOLLAND AND J. H. C. NAYLER (*Anal. Chem.*, 27 (1955) 971) as I,L,O.
S_6 = Butanol–acetic acid–water (4:1:5).
S_7 = Decalin–acetic acid–isopentanol (15:10:2) (W. GRASSMANN, H. HÖRMANN AND H. ENDRES, *Chem. Ber.*, 86 (1953) 1477).
S_8 = Decalin–10% acetic acid–isopentanol (15:10:4) (as for S_7).
S_9 = *tert.*-Pentyl alcohol–pH 6 phthalate buffer (S. BLACKBURN AND A. G. LOWTHER, *Biochem. J.*, 48 (1951) 126).

Paper: P_1 = Whatman No. 1.
P_2 = Whatman No. 4.

Detection: D_1 = Ninhydrin reagent.
D_2 = *p*-Nitrobenzoyl chloride–pyridine reagent (J. C. SHEEHAN, H. G. ZACHAU AND W. B. LAWSON, *J. Am. Chem. Soc.*, 80 (1958) 3349).
D_3 = U.V. light.
D_4 = Periodate–anisidine reagent (F. D. BRAGG AND L. HOUGH, *J. Chem. Soc.*, (1958) 4050).

Compound	R_F									Colour*			
	S_1P_1	S_2P_1	S_3P_1	S_4P_1	S_5P_1	S_6P_1	S_7P_1	S_8P_1	S_9P_2	D_1	D_2	D_3	D_4
Threonine	0.43	0.31								p			
α-Aminobutyric acid	0.63	0.31								p			
4-Oxopipecolic acid	[illegible]	[illegible]								[illegible]			
Proline	0.81	0.40								y			
Phenylglycine	0.74	[illegible]								p	r		
DL-p-Dimethylamino-N-methylphenylalanine	0.93	0.09, 0.75	0.70	0.81	0.77	0.30				bn**	r		
3-Hydroxypicolinic acid	0.70	0.61											
DL-2-Amino-2-phenyl-ethanol										bn→p			+
2-(2,4-Dinitrophenylamino)-2-phenylethanol							0.47	0.86					
Tetrapeptide***									0.34	—		fe	
Pentapeptide†									0.77	—		fe	

* p = purple; o = orange; r = red; y = yellow; bn = brown; fe = fluorescence; + = positive result; — = negative result.
** Development above 80°.
*** Containing 3-hydroxypicolinic acid, α-aminobutyric acid, proline and threonine.
† Containing 3-hydroxypicolinic acid, α-aminobutyric acid, proline, threonine and p-dimethylamino-N-methylphenylalanine.

TABLE 68

R_F VALUES OF GLUCOSAMINE-1-PHOSPHATE AND N-ACETYLGLUCOSAMINE-1-PHOSPHATE

(G. G. [illegible], B. H. CHASE, G. W. KENNER AND A. TODD, *J. Chem. Soc.*, (1960) 4678)

Solvents: S_1 = Propanol–1% $(NH_4)_2SO_4$.
S_2 = Propanol–ammonia–water (6:3:1, v/v).
S_3 = 95% ethanol–1 *M* ammonium acetate (75:30).
Paper: Not given; ascending.
Impregnation: I_1 = 1% $(NH_4)_2SO_4$, then dried.
Detection: Not given.

Compound	R_F		
	S_1I_1	S_2	S_3
Glucosamine-1-phosphate	0.26	0.15	0.09
N-Acetylglucosamine-1-phosphate	0.49	0.22	0.19

TABLE 69

R_F VALUES OF SOME LANTHIONINE DERIVATIVES AND RELATED COMPOUNDS

(M. FRANKEL AND D. GERTNER, *J. Chem. Soc.*, (1961) 459, 463)

Solvent: 80% aqueous phenol.
Paper: Not given.
Detection: Not given.

Compound	R_F
N,N'-Dibenzyloxycarbonyl-meso-lanthionine diethyl ester	0.95
N-Benzyloxycarbonyl-lanthionine monobenzyl ester	0.90
N-Acetyl-lanthionine monomethyl ester	0.84–0.86
S-Benzyloxycarbonyl-DL-homocysteine	0.92

TABLE 70

R_F VALUES OF SOME L-LYSINE PEPTIDES

(B. BEZAS AND L. ZERVAS, *J. Am. Chem. Soc.*, 83 (1961) 719)

Solvent: Butanol–acetic acid–water–pyridine (Y. LEVIN, A. BERGER AND E. KATCHALSKI, *Biochem. J.*, 63 (1956) 308).
Paper: Whatman No. 1, ascending.
Detection: Ninhydrin.

Compound	R_F
N^{ε}-Glycyl-L-lysine	0.18
N^{ε}-L-Valyl-L-lysine	0.36
N^{ε}-L-Phenylalanyl-L-lysine	0.46
N^{ε}-L-Phenylalanyl-L-lysyl-L-tyrosine	0.82
N^{α}-L-Phenylalanyl-L-lysine	0.46
N^{α}-L-Phenylalanyl-N^{ε}-L-valyl-L-lysine	0.62

TABLE 71

R_F VALUES OF SOME ACETYLSERINE PEPTIDES

(L. BENOITON AND H. N. RYDON, *J. Chem. Soc.*, (1960) 3328)

Solvents: S_1 = *tert.*-Butanol–formic acid–water (70:15:15).
S_2 = *n*-Butanol–acetic acid–water (4:1:5).
S_3 = Phenol saturated with 10% sodium citrate.
Paper: Whatman No. 11.
Detection: D_1 = Ninhydrin reagent.
D_2 = Chlorine–starch–iodide procedure (H. N. RYDON AND P. W. G. SMITH, *Nature*, 169 (1952) 922).

Compound	R_F		
	S_1	S_2	S_3
DL-Serylglycylglycine	0.28	0.22	0.43
O-Acetyl-DL-serylglycylglycine	0.40	0.32	0.65
N-Acetyl-DL-serylglycylglycine	0.51	0.40	0.73
α-L-Aspartyl-O-acetyl-DL-serylglycylglycine	0.32	0.25	0.49
O-α-L-Aspartyl-N-acetyl-DL-serylglycylglycine	0.32	0.24	0.49

TABLE 72

R_F VALUES OF SOME PEPTIDES

(D. T. GISH, *J. Am. Chem. Soc.*, 82 (1960) 6329)

Solvent: Butan-1-ol–acetic acid–water–pyridine (30:6:24:20) (S. G. WALEY AND J. WATSON, *Biochem. J.*, 55 (1953) 328).
Paper: Whatman No. 3 MM.
Detection: D_1 = Ninhydrin reagent.
D_2 = *p*-Dimethylaminobenzaldehyde reagent (I. SMITH, *Nature*, 171 (1953) 43).
D_3 = Sakaguchi reagent (R. ACHER AND C. CROCKER, *Biochim. Biophys. Acta*, 9 (1952) 704).
D_4 = Hypochlorite–starch–KI test (S. C. PAN AND J. D. DUTCHER, *Anal. Chem.*, 28 (1956) 836).
D_5 = Chlorine–starch–KI test (H. N. RYDON AND P. W. G. SMITH, *Nature*, 169 (1952) 922).

Peptide	R_F	*Colour**				
		D_1	D_2	D_3	D_4	D_5
Lys·Pro·Ser·Pro·GluNH$_2$	0.18	+	—	—		
Lys·Pro·Ser·Pro·GluNH$_2$·Val·Thr	0.27	+	—	—		
Val·Arg	0.40	+	—	+		
Val·Thr·Val·Arg	0.51	+	—	+		
Ser·GluNH$_2$·Val·Try	0.65	y	+	—		
Pyroglu·Phe	0.65	y	+	—	+	
Pyroglu·Phe·Ser·GluNH$_2$·Val·Try	0.90	—	+	—		
Phe	0.61	+				—
Pyroglu	0.43	—				+

* y = yellow; + = positive; — = negative.

TABLE 73

R_F VALUES (RELATIVE) OF SOME PEPTIDES

(A. WITTER AND H. TUPPY, *Biochim. Biophys. Acta*, 45 (1960) 429)

Solvent: *n*-Butanol–pyridine–acetic acid–water (4:1:1:5, by vol.).
Paper: Schleicher & Schüll 2045 bM.
Detection: Ninhydrin.

*Compound**	*RPhe***	*Colour****
Cys-Pro	0.31	pu
Pro-Phe	1.21	y
Glu-Cys-Pro	0.08	v
Glu-Glu-Cys-Pro	0.13	v
Glu-Cys-Pro-Phe	0.22	v
Cys-Pro-Phe	0.37	p
Glu-Glu-Cys	0.05	v
Glu-Cys	0.08	pu
Glu-Glu	0.13	pu
Phe	1.00	v
Glu	0.19	v
Pro	0.43	y
2-Amino-2-carboxyethyl-mercaptosuccinic acid	0.08	

* Inferred sequence where peptide.
** R_F relative to that of phenylalanine.
*** pu = purple; y = yellow; v = violet; p = pink.

TABLE 74

R_F VALUES OF SOME PEPTIDES AND PEPTIDE DERIVATIVES

(D. THEODOROPOULOS AND J. GAZOPOULOS, *J. Chem. Soc.*, (1960) 3861)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Butan-2-ol–formic acid–water (1:3:2).
S_3 = Butan-1-ol–acetic acid–pyridine–water (15:3:10:12).
Paper: Whatman No. 1.
Detection: 0.1% ninhydrin in ethanol.

Compound	R_F		
	S_1	S_2	S_3
L-Isoleucyl-1(or 3)-benzyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester dihydrobromide	0.85	0.77	
L-Valyl-L-tyrosine methyl ester hydrochloride			0.91
L-Isoleucyl-1(or 3)-benzyl-L-histidyl-L-prolyl-L-phenylalanine		0.76	
L-Isoleucyl-1(or 3)-benzyl-L-histidyl-L-prolyl-L-phenylalanine benzyl ester ditoluene-*p*-sulphonate			0.95

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